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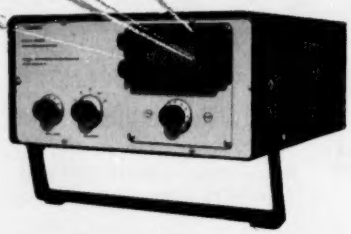

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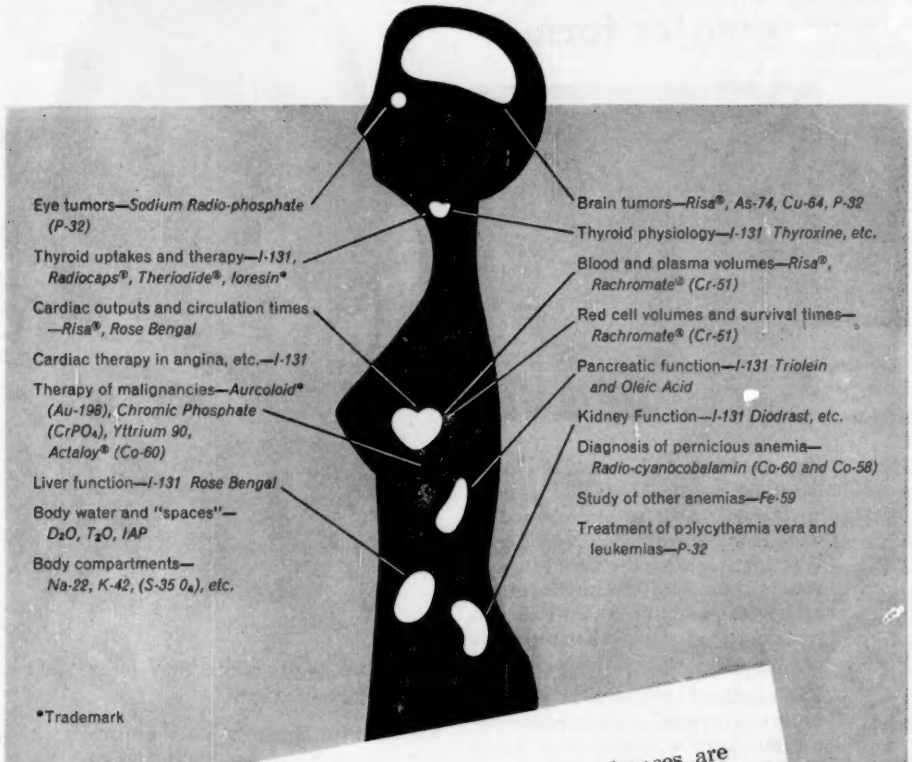
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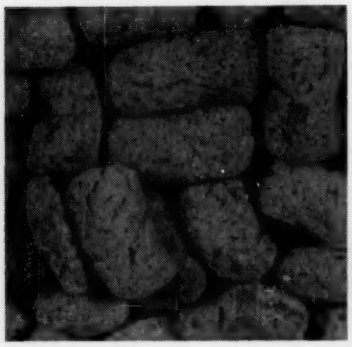

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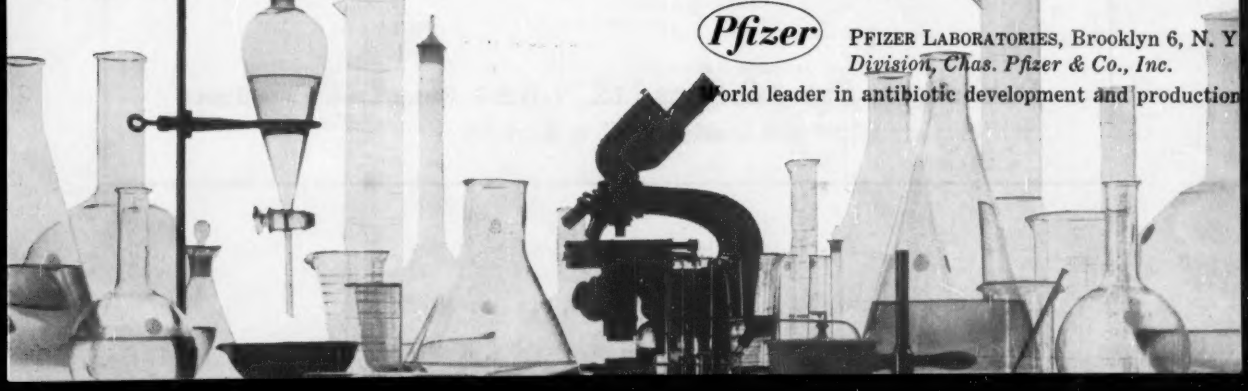

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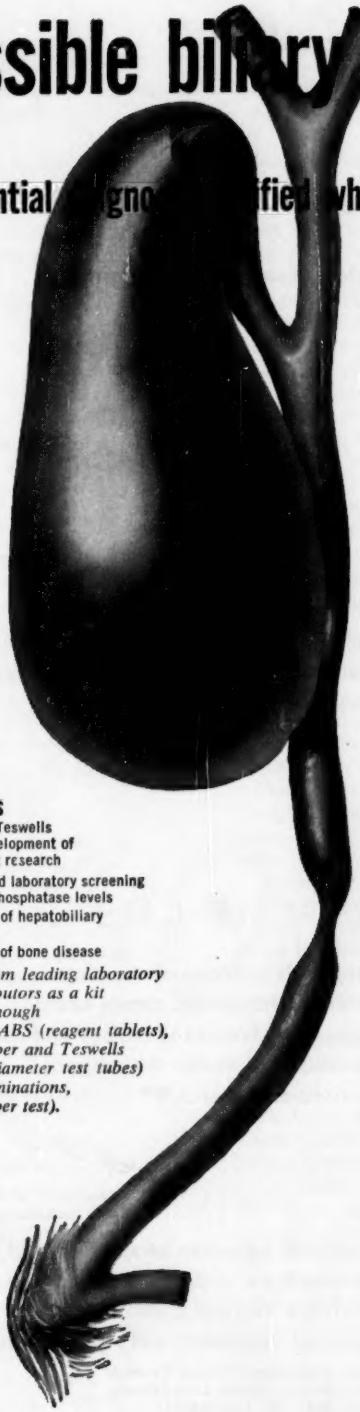

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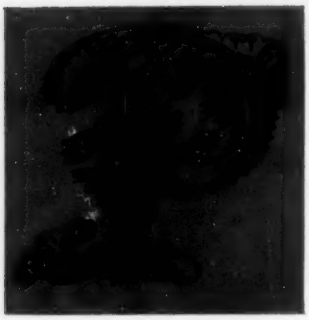

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THE EFFECTS OF EXPERIMENTAL HEPATOBILIARY DISEASE ON CERTAIN ASPECTS OF TOCOPHEROL METABOLISM IN THE RAT[1]

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In a previous study (1) it was found that the plasma tocopherol level in patients with hepatocellular disease tended to be low and relatively unresponsive to an oral test dose of tocopheryl acetate. However, repeated doses raised the concentration in a normal manner, while the fraction recovered in the feces was even smaller than in normal subjects (2, 3), suggesting that intestinal absorption was not impaired. One possible explanation for these findings was that the tissue stores of tocopherol were depleted as the consequence of an antecedent dietary deficiency, and that the low concentrations in the feces and the relatively flat tolerance curves were indicative of greater absorption and more rapid transfer of tocopherol from the intestinal tract to the tissues. Consistent with this interpretation was the observation that the plasma levels in liver disease did not differ significantly from those in randomly selected patients with a variety of other disorders in which malnutrition could have been a factor. Since there was no loss of tocopherol during its incubation with feces, aspirated jejunal contents or pure cultures of enteric bacteria (3), it appeared unlikely that the abnormalities noted in patients with hepatocellular damage were due to increased tocopherol degradation in the intestinal tract. However, the report of Rosenkrantz, Milhorat, and Farber (4) that a significant fraction of tocopherol is excreted as tocopherylquinone, an oxidative product which is not detectable in conventional analyses based on the Emmerie-Engel reaction, necessitated further investigation of this possibility. Accordingly, simultaneous determinations of fecal tocopherol and tocopherylquinone were carried out on three-day stool collections before and after a one Gm. oral test dose of tocopheryl acetate, using a method described by these authors (5). It was found that neither normal nor cirrhotic subjects excreted a significant amount of tocopherylquinone (6), an observation consistent with the impression that the low levels of fecal tocopherol in liver disease were not due to increased degradation in the intestinal tract.

As in the case of hepatocellular disease, patients with partial occlusion of the common bile duct exhibited low levels of plasma tocopherol and relatively flat tolerance curves, and excreted only a small fraction of ingested tocopherol, but, in contrast, showed no increase in plasma concentration when repeated large doses of tocopherol were administered (3). It was difficult to reconcile these findings with either an absorptive defect or a deficiency state leading to greater absorption and more rapid transfer of tocopherol from the intestinal tract to depleted tissue depots. Since Popper, Dubin, Steigmann, and Hesser (7) had reported high levels of plasma tocopherol in both obstructive and hepatocellular jaundice, the possibility was considered that the low output of tocopherol in the feces in these conditions was due to interference with its re-excretion in the bile. This was investigated by measuring the output of tocopherol in T-tube bile from patients with biliary fistulae, and in aspirated duodenal contents of cirrhotic and normal subjects with an occlusive balloon in the jejunum (3). It was found that the amount recovered constituted only a small fraction of the total fecal tocopherol, and did not increase during the absorption of tocopherol from the intestine. Moreover, the excretion in normal and cirrhotic subjects did not differ, so that biliary retention did not appear to be a significant factor in the reduced fecal excretion of tocopherol in liver disease, a conclusion supported by the accompanying low levels of tocopherol found in the

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plasma. In contrast to the experience of Popper and his associates (7), no high levels were encountered in either hepatocellular or obstructive jaundice, and there was no correlation between the plasma concentration of tocopherol and that of either bilirubin or cholesterol (3).

The results of these studies on the effects of hepatobiliary disease on the plasma tocopherol level and on the absorption and storage of tocopherol were considered inconclusive, and in some ways contradictory. No doubt some of the factors that could not be controlled or evaluated accurately, such as the nutritional status, the degree of bile stasis, and the severity of the hepatocellular injury, affected the results. It was considered worth while, therefore, to reinvestigate the problem in animals under more uniform conditions. The following is a report of such studies in rats subjected to ligation of the bile duct, bile fistula and acute and chronic carbon tetrachloride-induced hepatic injury.

METHODS

Animals. Male Sprague-Dawley rats, raised on an *ad libitum* diet of Purina Laboratory Chow containing 0.035 mg. of tocopherol per Gm., were subjected to the following procedures when they attained a weight of 200 to 300 Gm.:

1) Ligation of the bile duct. Under Nembutal® anesthesia, the bile duct was doubly ligated and transected as close to the hilum of the liver as possible. Special care was taken to avoid injury to the pancreas. Post-operatively, each animal received 10 ml. of saline subcutaneously to insure adequate hydration.

2) Bile fistula. Under Nembutal® anesthesia, a polyethylene catheter with a bore of 0.58 mm. was introduced into the bile duct and tied in place approximately 1 cm. from the hilum of the liver and proximal to a double ligature occluding the distal segment of the duct. The free end of the catheter was then threaded subcutaneously through a hollow needle to the nape of the neck where it was allowed to drain into a rubber finger cot lying on the back and held in place by a protective plastic shield sutured to the skin, as described by Hoffbauer, Watson, and Schwartz (8). The open end of the finger cot was sealed with a cork through which the catheter entered, while the tip emptied into a length of rubber tubing containing a removable glass plug to permit daily drainage. The water-tight sac thus created was sufficiently large to accommodate a 24 hour bile collection without tension, so that no hyperbilirubinemia ensued. Post-operatively, 10 ml. of saline was administered subcutaneously. Thereafter, normal saline was substituted for the drinking water to compensate for electrolyte losses in the bile. Animals maintained on this regimen tolerated biliary drainage for the seven day experimental period without apparent ill effects.

3) Acute hepatic injury. Three subcutaneous injections of carbon tetrachloride in doses of 0.15 ml., 0.10 ml., and 0.05 ml. per 100 Gm. of body weight were given at two day intervals to insure the presence of acute hepatic necrosis throughout the experimental period. This was confirmed histologically. A number of animals died, but the survivors used in subsequent studies appeared to be in a good state of health.

4) Chronic hepatic injury and cirrhosis. Carbon tetrachloride was injected subcutaneously at the rate of 0.05 ml. per 100 Gm. of body weight twice weekly for five months. Histological examination at this time revealed the presence of a finely granular cirrhosis. The mortality rate in this group was high, but the surviving animals used in subsequent tocopherol studies appeared healthy, had no signs of jaundice or ascites, and exhibited a reasonably rapid growth rate, attaining an average weight of 438 Gm., representing a gain of approximately 180 Gm.

Tocopherol studies. The acute effects of these procedures on the serum tocopherol concentration were measured daily for a period of one week in samples of blood obtained from a tail vein.

The method of evaluating changes in tocopherol absorption, transport and storage was based on a study of the serum tocopherol responses to test doses of tocopheryl acetate administered either (a) orally in the form of a suspension in dilute alcohol, (b) orally in the form of a stable emulsion, or (c) intramuscularly in the form of a stable emulsion. Serum tocopherol concentration was estimated at 0, 2, 4, 6, 8, 10, 12, 24, 48, 72 and 96 hours, each of the three tolerance tests being carried out in an individual group of six animals. The curves obtained were then analyzed statistically in terms of maximum and mean increase in concentration over the control level, and were compared with each other in each of the four experimental groups, and with those obtained in control animals.

The oral suspension was prepared by vigorously mixing a 10 per cent solution of *dl*-alpha-tocopheryl acetate[3] in 95 per cent ethyl alcohol (w/v) with 40 times its volume of water in a small test tube with the aid of a fine needle and syringe, thus yielding a final alcohol concentration of approximately 2.3 per cent. The required amount was then promptly drawn into a syringe and instilled into the stomach through a metal cannula passed down the esophagus.

The oral emulsion,[4] which was a 3.3 per cent (w/v) stable dispersion of *d*-alpha-tocopheryl acetate in a 10 per cent aqueous solution of Tween 80, was administered in the same fashion. It has been shown that stabilization in the Tweens enhances the absorption of tocopheryl ace-

[3] Obtained from Hoffman-LaRoche, Inc., Nutley, N. J., through the courtesy of Dr. Robert J. Floody.

[4] Obtained from the United States Vitamin Corporation, New York, N. Y., through the courtesy of Dr. Louis Freedman.

tate in normal individuals (9), and, at least in the case of vitamin A, is capable of abolishing the absorptive defect in patients with steatorrhea (10). For that reason it was hoped that a comparison of the tolerance curves obtained with the emulsion and suspension might reveal any impairment of intestinal absorption related to disturbances in bile flow.

The intramuscular preparation [4] was a stabilized 2.5 per cent emulsion of *dl*-alpha-tocopheryl acetate in water containing 5 per cent Tween 20, 2 per cent procaine HCl and 0.5 per cent chlorbutanol (all values expressed as w/v). Since preparations of this type are known to be absorbed rapidly from muscle (11), study of the serum concentrations following an injection appeared to provide an indirect method for measuring changes in the rate of tocopherol utilization and/or transfer to the tissues.

The tolerance tests were carried out (a) 72 hours after ligation or external drainage of the bile duct to avoid any immediate post-operative effects, and to rid the intestinal tract of previously secreted bile, (b) 48 hours after the initial injection of carbon tetrachloride to coincide with the point of maximum hepatic necrosis; the second injection was given at the same time as the tocopheryl acetate, while the third was administered 48 hours later to perpetuate the hepatic injury throughout the course of the 96 hour observation period, and (c) two weeks after the cessation of a five month series of carbon tetrachloride injections to avoid the complicating effects of acute hepatic injury related to the most recent injections.

In the animals with a fistula the bile was collected daily and analyzed for its tocopherol content.

Other studies. Following completion of the tolerance tests, the animals were sacrificed and examined for evidence of recanalization or rupture of the bile duct in those with ligated ducts, and for evidence of biliary obstruction or leakage in those with a biliary fistula. If any of these complications were found, the experimental results were discarded.

Serum bilirubin determinations were carried out serially in each of the experimental groups to investigate the possible relationship between the serum tocopherol level and bile retention. The same samples were analyzed for total cholesterol, since previous investigators (12) had noted a direct relationship between the concentration of tocopherol and cholesterol in a number of conditions. It should be pointed out that these studies were not carried out in the animals undergoing the tocopherol tolerance tests. Unfortunately, the amount of blood required was too large to permit simultaneous serial analyses of tocopherol, bilirubin and cholesterol in the same animals.

Chemical methods. Serum tocopherol determinations were carried out in triplicate by the micromethod of Quaife, Schrimshaw, and Lowry (13). The three values checked closely, as evidenced by the fact that the standard deviation of the means for all individual samples analyzed was only ± 0.04 mg. per cent.[5] Moreover, parallel analyses of 15 pairs of serum revealed that the results were in close agreement with those obtained by the macromethod of Quaife and Biehler (14).

Estimations of serum bilirubin and cholesterol were carried out by microscale modifications of the Malloy-Evelyn (15) and Saifer-Kammerer (16) methods, respectively.

[5] $\sqrt{\frac{\text{Sum of (deviations from the mean of each sample)}^2}{\text{Sum of (n–1 for each sample)}}}$.

RESULTS

Control studies

The serum tocopherol concentration in rats of identical age and sex and raised on a uniform diet varied over a wide range (Table I). However, the hourly and daily fluctuations in individual animals were relatively small (Table II), suggesting that the variations within the group were not due to postprandial changes in serum concentration related to *ad libitum* feeding.

Following the oral administration of *dl*-alpha-tocopheryl acetate in the form of an unstable suspension in 2.3 per cent ethanol, the serum tocopherol level rose significantly, reaching a peak in 8 to 12 hours, and returned to the initial concentration, or slightly above, in 24 to 48 hours. The magnitude of the response varied with the amount of tocopherol administered, approaching a ceiling at a dose of 10 mg. of free tocopherol per 100 Gm. of body weight. Presumably, absorption was maximal at this level, so that the 10 mg. dose was employed in all subsequent experiments. In comparing the effects of different preparations and routes of administration, the curve obtained with the oral suspension of tocopheryl acetate was adopted as a standard of reference. To facilitate their comparison, the curves were analyzed statisti-

TABLE I

Serum tocopherol level in rats under varying experimental conditions

Experimental group	No. of animals	Mean ± S.D.*	Range
		mg. %	*mg. %*
Controls	64	0.39 ± 0.23	0.03–1.05
Ligation of bile duct			
72 hours	27	0.61 ± 0.45†	0.00–1.38
7–11 days	24	0.42 ± 0.18	0.00–0.65
Bile fistula—72 hours	18	0.90 ± 0.47†	0.11–1.72
Carbon tetrachloride			
48 hours	24	0.78 ± 0.48†	0.09–1.93
5 months	22	0.46 ± 0.23	0.09–0.98

* S.D. refers to standard deviation.

† Statistically significant differences: ligation of bile duct (72 hours) > controls ($p < 0.02$); bile fistula > controls ($p < 0.01$); carbon tetrachloride (48 hours) > controls ($p < 0.01$).

TABLE II

Fluctuations in serum tocopherol concentration of rats maintained on an ad libitum stock diet

Rat no.	Serial serum tocopherol determinations						Mean
	1	2	3	4	5	6	
	Hourly intervals						
	mg. %						mg. %
1	0.54	0.55	0.48	0.50	0.30	0.35	0.45
2	0.55	0.51	0.58	0.44	0.43	0.45	0.49
3	0.29	0.32	0.45	0.32	0.35	0.33	0.34
4	0.48	0.31	0.47	0.31	0.31	0.34	0.37
5	0.32	0.29	0.30	0.16	0.23	0.22	0.25
6	0.28	0.28	0.27	0.26	0.25	0.19	0.26
Standard deviation of the observations in the group* = ±0.07 mg. per cent							
	Daily intervals						
7	1.05	0.68	0.68	0.81	0.99	0.86	0.85
8	0.66	0.68	0.75	0.73	1.04	0.77	0.77
9	0.85	0.78	0.69	0.74	0.98	0.86	0.82
10	0.88	0.44	0.56	0.74	0.81	0.76	0.70
11	0.59	0.66	0.65	0.68	1.14	1.02	0.79
12	0.84	0.83	0.86	0.94	0.95	0.88	0.88
13	0.32	0.28	0.18				0.26
14	0.29	0.48	0.40				0.39
15	0.54	0.58	0.45				0.52
16	0.30	0.29	0.44				0.34
17	0.36	0.31	0.47				0.38
18	0.33	0.29	0.53				0.38
19	0.52	0.57	0.59				0.56
Standard deviation of the observations in the group* = ±0.13 mg. per cent							

* $\sqrt{\frac{\text{Sum of (deviations from the mean of each subject)}^2}{\text{Sum of (n−1 for each subject)}}}$.

cally in terms of the maximum and mean increases in serum tocopherol concentration over 24 and 96 hour periods. The latter were calculated from the areas under the curves. Since the results obtained by all methods of analysis were in close agreement, only those pertaining to the maximum increase in concentration are presented.

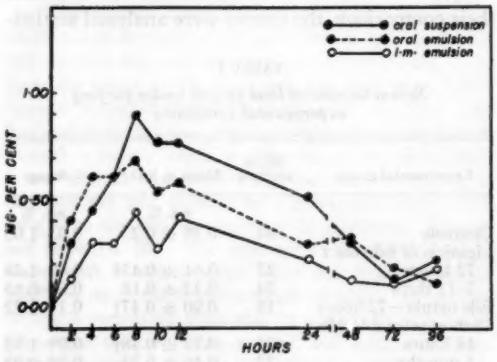

FIG. 1. INCREASE IN SERUM TOCOPHEROL IN RESPONSE TO THE ADMINISTRATION OF TOCOPHERYL ACETATE TO CONTROL RATS

Values shown represent the means for groups of six animals each.

As might be expected in control animals with an intact digestive mechanism, homogenization of the tocopheryl acetate to produce a stable dispersion of microscopic particles did not enhance its rate of absorption from the intestinal tract (Figure 1, Table III). However, it did permit its absorption from muscle, although the rate of absorption, judging from its effect on serum tocopherol, was significantly lower than that in the intestinal tract (Figure 1, Table III).

Biliary obstruction

Following ligation of the bile duct, the serum tocopherol concentration rose transiently, reaching a peak in 72 hours, and returned to the control level by the fifth day (Figure 2). This was not a constant finding, but in the 27 experiments carried out the mean of the concentrations found at 72 hours was significantly higher than that of the

controls, while the mean of the values found between the fifth and eleventh days was within normal limits (Table I). The transient character of the rise in the face of persistent complete biliary obstruction, as evidenced by the progressive increase in serum bilirubin (Figure 2), made it unlikely that it was due to retention of tocopherol normally excreted in the bile. The possibility that it was related to the hypercholesteremia that accompanied it was suggested by the striking similarity between the concentration curves of the two lipids (Figure 2). According to Byers, Friedman, Biggs, and Gunning (17), the hypercholesteremia that follows ligation of the bile duct in the rat is due primarily to an accumulation of cholate in the blood, leading to an increase in the cholesterol-binding capacity of the serum proteins and a consequent retention of cholesterol. Unfortunately, the period of observation in the experiments carried out by these investigators was limited to 72 hours, so that no data are available on the serum cholate level for the subsequent period during which the serum cholesterol was found to fall both in the present study and that of Chanutin and Ludewig (18). Although the serum cholate concentration tends to remain elevated following bile duct ligation in most animals (19, 20), it is conceivable that the rat is an exception, and that the rise and fall in the concentrations of both cholesterol and tocopherol were due to parallel fluctuations in the cholate level. Since the rat is incapable of synthesizing tocopherol, any increment in the serum must have been derived from either the diet or the tissues. Accordingly, the possibility had to be considered that the fluctuations in serum tocopherol were due to alterations in intestinal absorption, or to changes in the output or uptake of tocopherol by the tissues, and particularly by the liver, which contains one of the largest and most readily mobilized depots of tocopherol (21).

As is evident from Figure 3 and Table III, orally administered tocopheryl acetate in suspension raised the serum tocopherol concentration only slightly. In emulsified form it had a somewhat

TABLE III

Maximum increase in serum tocopherol concentration following the administration of tocopheryl acetate *

Experimental group‡	Mean ± S.D.	Statistically significant differences†					
		Comparison with control animals			Comparison with animals in the same experimental group		
		Oral suspension	Oral emulsion	I.M. emulsion	Oral suspension	Oral emulsion	I.M. emulsion
	mg. %						
Controls							
Oral suspension	0.98 ± 0.34						
Oral emulsion	0.76 ± 0.13						
I.M. emulsion	0.51 ± 0.16				$p < 0.02$	$p < 0.02$	
Ligated bile duct							
Oral suspension	0.12 ± 0.05	$p < 0.01$					
Oral emulsion	0.32 ± 0.17	$p < 0.01$	$p < 0.01$				
I.M. emulsion§	4.24 ± 1.20	$p < 0.01$		$p < 0.01$	$p < 0.01$	$p < 0.01$	
Bile fistula							
Oral suspension	0.24 ± 0.21	$p < 0.01$					
Oral emulsion	0.76 ± 0.90						
I.M. emulsion§	1.10 ± 0.38			$p < 0.01$	$p < 0.01$		
CCl_4 (acute)							
Oral suspension	0.50 ± 0.44						
Oral emulsion	1.51 ± 0.73		$p < 0.05$		$p < 0.02$		
I.M. emulsion	1.03 ± 0.21			$p < 0.01$	$p < 0.05$		
CCl_4 (chronic)							
Oral suspension	0.61 ± 0.14						
Oral emulsion	1.18 ± 0.63						
I.M. emulsion	0.53 ± 0.33					$p < 0.05$	

* Dose used equivalent to 10 mg. free tocopherol per 100 Gm. body weight.
† Determined by Students' "t" test.
‡ Six animals per group, except: CCl_4 (acute), oral emulsion, seven; CCl_4 (chronic), oral suspension, four.
§ Difference statistically significant, $p < 0.01$.

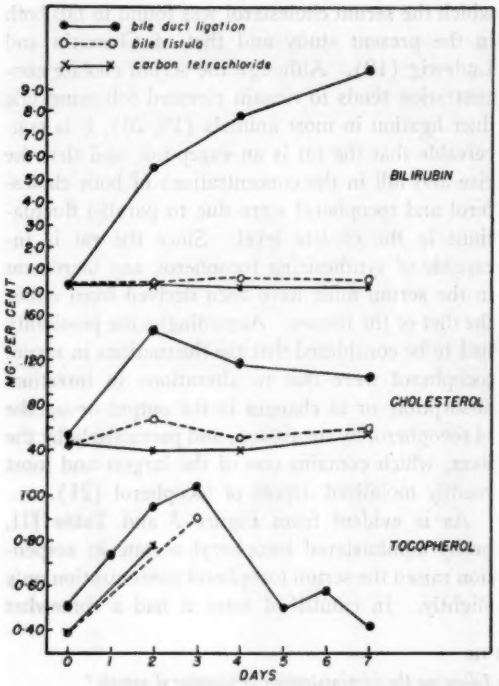

FIG. 2. CHANGES IN SERUM BILIRUBIN, CHOLESTEROL, AND TOCOPHEROL FOLLOWING LIGATION OF THE BILE DUCT, EXTERNAL DRAINAGE OF THE BILE DUCT, OR THE ADMINISTRATION OF CARBON TETRACHLORIDE

Values shown represent the means for groups of six animals each, except in the case of the serum tocopherol levels in animals with a bile fistula or acute carbon tetrachloride intoxication, which were derived from the data in Table I.

greater effect, but the difference was not statistically significant. Moreover, in neither case was the response equal to that in the controls, suggesting that tocopherol absorption from the intestinal tract was impaired, presumably due to the absence of bile. It is highly improbable, therefore, that the increment in serum tocopherol following ligation of the bile duct was of dietary origin, particularly since the diet provided only 0.14 to 0.18 mg. per 100 Gm. of body weight.

In contrast, the intramuscular administration of tocopheryl acetate produced a striking increase in serum tocopherol that greatly exceeded the response in any of the controls (Figure 3, Table III). Since the level remained elevated for a longer period than the hypercholesteremia, it is unlikely that a high blood cholate concentration was a major factor in the retention of tocopherol in the serum.

To investigate further the possibility that ligation of the bile duct interrupted a normal pathway of tocopherol excretion, or reduced the capacity of the liver to store tocopherol, similar studies were carried out in animals with bile fistula and with carbon tetrachloride-induced acute hepatic injury.

Bile fistula

The serum tocopherol level rose transiently following the creation of a bile fistula, reaching a peak in 72 hours (Table I). Since there was no accompanying hypercholesteremia (Figure 2), it is unlikely that this effect was due to an increase in blood cholate.

Orally administered tocopheryl acetate in suspension raised the serum concentration less than in the controls (Figure 4, Table III), suggesting that intestinal absorption was impaired. However, the defect appeared to be less severe than in animals with biliary obstruction since homogenization of the tocopherol in Tween 80, which was in-

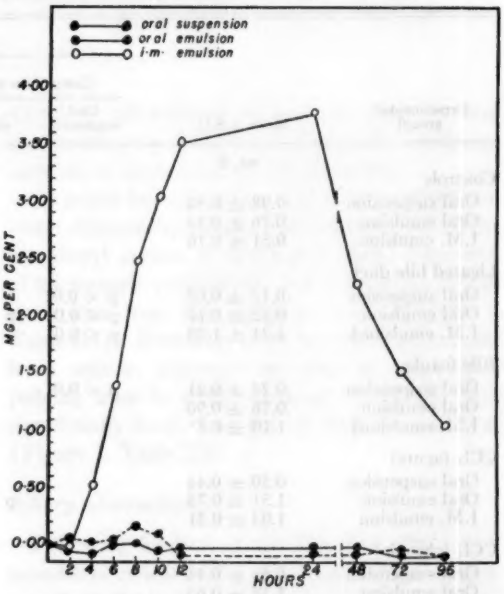

FIG. 3. INCREASE IN SERUM TOCOPHEROL IN RESPONSE TO THE ADMINISTRATION OF TOCOPHERYL ACETATE 72 HOURS FOLLOWING LIGATION OF THE BILE DUCT

Values shown represent the means for groups of six animals each.

TABLE IV

Biliary excretion of tocopherol before and after the administration of tocopheryl acetate *

Tocopheryl acetate preparation	Days before or after tocopheryl acetate administration						
	−3	−2	−1	+1	+2	+3	+4
	Tocopherol concentration in bile, *mg.* % †						
Oral suspension	0.43	0.26	0.25	0.14	0.16	0.20	0.19
Oral emulsion	0.47	0.28	0.23	0.26	0.26	0.24	0.33
I.M. emulsion	0.37	0.50	0.46	0.42	0.41	0.30	0.28
	Amount of tocopherol excreted, *mg.*†						
Oral suspension	0.05	0.03	0.03	0.02	0.02	0.03	0.02
Oral emulsion	0.06	0.03	0.03	0.03	0.03	0.03	0.04
I.M. emulsion	0.03	0.05	0.05	0.05	0.05	0.03	0.03

* Dose used equivalent to 10 mg. free tocopherol per 100 Gm. body weight.
† Mean values for groups of six animals each.

effective in the latter, permitted normal absorption in animals with a bile fistula, as evidenced by a serum response that was equal to that of the controls (Table III). Although ligation and transection of the bile duct were carried out as close to the porta hepatis as possible to avoid injury or obstruction to the pancreatic ducts, which enter the bile duct along most of its length, it is conceivable that the inflammatory reaction and local pressure effects associated with elongation and distension of the proximal occluded segment of the bile duct interfered with pancreatic function, thus compounding the absorptive defect due to the absence of bile.

Emulsified tocopheryl acetate produced a greater rise in serum tocopherol concentration when injected intramuscularly than when administered orally (Figure 4, Table III). This difference could not have been due solely to the more rapid absorption of tocopherol from muscle, since the increase in serum concentration was even larger than that observed following a similar injection in normal animals (Table III). Inasmuch as hypercholesteremia and bile retention could be excluded as factors, a decrease in the uptake of tocopherol by the liver appeared to be an attractive possibility worthy of further investigation.

It is noteworthy that the increase in serum tocopherol following an intramuscular injection of tocopheryl acetate was significantly greater in animals with obstruction of the bile duct than in those with a biliary fistula (Table III). Hypercholesteremia and/or cholate retention could have been responsible for this difference, although the possibility that the mechanism for the uptake of tocopherol by the liver was more severely impaired under conditions of biliary obstruction could not be excluded. As is evident from the data in Table IV, retention in the serum of tocopherol normally excreted in the bile could not have been a significant factor. Thus, it will be noted that the amount of tocopherol excreted in the bile constituted only a negligible fraction of the intake, and did not increase significantly when large doses of tocopherol were administered either orally or intramuscularly.

Carbon tetrachloride-induced acute hepatic injury

At the height of the hepatic injury 48 hours after the administration of carbon tetrachloride,

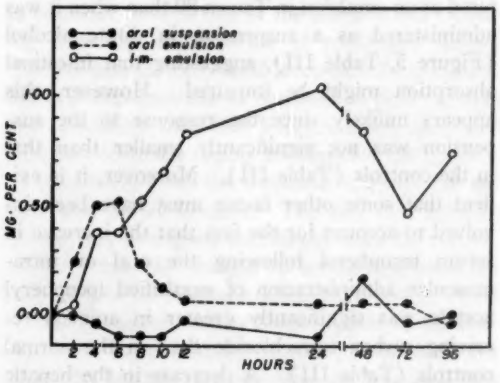

FIG. 4. INCREASE IN SERUM TOCOPHEROL IN RESPONSE TO THE ADMINISTRATION OF TOCOPHERYL ACETATE 72 HOURS FOLLOWING THE CREATION OF AN EXTERNAL BILE FISTULA

Values shown represent the means for groups of six animals each.

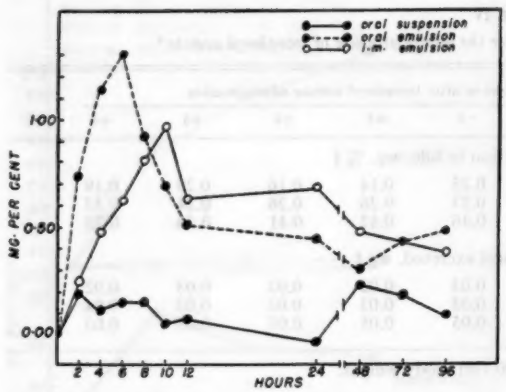

FIG. 5. INCREASE IN SERUM TOCOPHEROL IN RESPONSE TO THE ADMINISTRATION OF TOCOPHERYL ACETATE 48 HOURS FOLLOWING CARBON TETRACHLORIDE INTOXICATION

Values shown represent the means for groups of six animals each, except in the case of those given the oral emulsion, of which there were seven.

the serum tocopherol concentration was significantly higher than that in the controls (Table I). Since there was no accompanying change in the serum bilirubin or cholesterol (Figure 2), it is unlikely that the increase in serum tocopherol was a retention phenomenon related to the suppression of bile secretion or the accumulation of blood cholate.

Tocopheryl acetate raised the serum tocopherol to a significantly higher level when it was administered as an emulsion in Tween 80 than when it was administered as a suspension in dilute alcohol (Figure 5, Table III), suggesting that intestinal absorption might be impaired. However, this appears unlikely since the response to the suspension was not significantly smaller than that in the controls (Table III). Moreover, it is evident that some other factor must have been involved to account for the fact that the increase in serum tocopherol following the oral or intramuscular administration of emulsified tocopheryl acetate was significantly greater in animals receiving carbon tetrachloride than in the normal controls (Table III). A decrease in the hepatic uptake of tocopherol as a consequence of injury would best explain these findings, and might, in part at least, account for the supranormal responses to intramuscular tocopherol observed in animals with ligation of the bile duct and biliary fistula.

The question arises whether the transient elevations of serum tocopherol observed following ligation of the bile duct, creation of a bile fistula, and the administration of carbon tetrachloride also were due to a decrease in the hepatic uptake of tocopherol entering the serum from the intestinal tract or other tissues. This appears unlikely, since the progressive hepatic damage seen under these conditions might be expected to produce a sustained rather than a transient retention of tocopherol in the serum. The alternative possibility, that hepatic injury resulted in the release of tocopherol from the liver itself, appears more plausible, and is consistent with the observation that the increase in serum tocopherol was independent of bile retention or elevation of the serum cholesterol level. The recent report of Yamamoto, Okuda, and Chow (22) that the administration of carbon tetrachloride increases the release of vitamin B_{12} from the liver and raises its concentration in the serum lends credence to this interpretation.

Carbon tetrachloride-induced chronic hepatic injury and cirrhosis

The mean serum tocopherol concentration in rats given carbon tetrachloride for a period of five months and then allowed to recover for two weeks did not differ significantly from that of the controls (Table I). This suggests that the tissue stores of tocopherol were not depleted and, hence, that chronic hepatic injury and cirrhosis had not affected the absorption of dietary tocopherol, an impression that was confirmed by the results of oral tolerance tests. These revealed that the rise in serum tocopherol following the administration of tocopheryl acetate, in the form of either a suspension or an emulsion, was equal to that in the controls (Figure 6, Table III). Also, judging from the normal serum response to intramuscularly injected tocopheryl acetate (Table III), it is evident that the hepatic uptake of tocopherol was not impaired.

COMMENT

The concentration of tocopherol in the serum depends on the relative rates at which tocopherol enters and leaves the blood. Thus, the level at which equilibrium is established may be influenced by the dietary intake of tocopherol, its rate of ab-

sorption from the intestinal tract, and the capacity of the tissues to store, utilize, and release tocopherol. As far as is known, endogenous synthesis is not a factor, and, as shown previously, the negligible losses of tocopherol in the bile and urine are of little consequence (2). The binding capacity of the serum proteins may affect the amount of tocopherol retained, and may govern the maximum concentration attainable in the serum. However, it is doubtful that a reduction in the binding capacity is ever responsible for low serum concentrations, since even at low levels it is possible to raise the concentration very considerably with an intramuscular injection of tocopherol. Considering the multiplicity of factors involved, it is not surprising that the serum concentration of tocopherol varies over a wide range, even in animals raised under identical conditions.

Since the concentration of tocopherol in the liver is more responsive to changes in the dietary intake than that in any of the other tissues (21), it must be assumed that the liver is capable of storing and mobilizing tocopherol more readily, and, hence, may play a significant role in the regulation of its concentration in the serum. It is reasonable to postulate, therefore, that the transient rise in concentration following experimental hepatocellular injury or surgical manipulation of the bile duct, and the abnormally high levels attained under these conditions following an intramuscular injection of tocopherol, are attributable to alterations in the mechanism for the release and uptake of tocopherol by the liver. In the case of biliary obstruction, an increase in serum binding capacity, possibly related to the retention of cholesterol or cholate, may be a contributory factor.

It is evident from these studies that, in the rat at least, complete interruption of bile flow into the intestinal tract, as a consequence of either ligation or external drainage of the bile duct, interferes with the absorption of tocopherol, while acute or chronic carbon tetrachloride-induced hepatocellular injury does not. No doubt the absence of bile retention in the latter accounts for this difference. Accordingly, it might be anticipated that other forms of hepatic damage that give rise to jaundice would produce a similar absorptive defect. However, on the basis of studies in man, it would appear that incomplete suppression of bile flow does not affect tocopherol absorption. Thus, as previously reported (2, 3), patients with partial biliary obstruction, acute viral hepatitis, and Laennec's cirrhosis, in whom bile excretion is diminished but not abolished, do not show excessive losses of tocopherol in the feces. Indeed, the fraction of ingested tocopherol excreted is even smaller than in normal subjects. Since there is no evidence of increased tocopherol destruction in the intestinal tract of such individuals, it must be assumed that a larger fraction is absorbed, possibly in response to depletion of the tissue stores resulting from the malnutrition of prolonged illness. The low plasma tocopherol levels and the relatively flat oral tolerance curves observed in such patients are consistent with this interpretation, and suggest that when the depots are depleted the transfer of tocopherol from the intestinal tract to the tissues may be accelerated. The fact that animals maintained in a good nutritional state do not exhibit comparable changes in serum concentration and tocopherol tolerance when subjected to acute or chronic hepatic injury lends support to the concept that hepatocellular disease *per se* does not alter the serum concentration or intestinal absorption of tocopherol.

It is evident that malnutrition and impairment of absorption due to the absence of bile in the intestinal tract may ultimately lower the serum tocopherol level. However, the retention of bile,

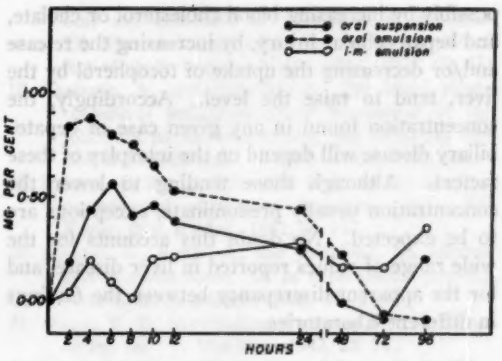

FIG. 6. INCREASE IN SERUM TOCOPHEROL IN RESPONSE TO THE ADMINISTRATION OF TOCOPHERYL ACETATE TO ANIMALS GIVEN BIWEEKLY INJECTIONS OF CARBON TETRACHLORIDE FOR FIVE MONTHS AND THEN ALLOWED TO RECOVER FOR TWO WEEKS

Values shown represent the means for groups of six animals each, except in the case of those given the oral suspension, of which there were only four.

possibly by increasing blood cholesterol or cholate, and hepatocellular injury, by increasing the release and/or decreasing the uptake of tocopherol by the liver, tend to raise the level. Accordingly, the concentration found in any given case of hepatobiliary disease will depend on the interplay of these factors. Although those tending to lower the concentration usually predominate, exceptions are to be expected. No doubt this accounts for the wide range of values reported in liver disease, and for the apparent discrepancy between the findings in different laboratories.

SUMMARY

The effects of biliary obstruction, external drainage of the bile duct, and acute and chronic carbon tetrachloride-induced hepatocellular injury on the serum concentration of tocopherol have been investigated in the rat both before and after the administration of oral and intramuscular test doses of tocopheryl acetate. It has been found that in well nourished rats (a) obstruction or external drainage of the bile duct interferes with the intestinal absorption of tocopherol, while acute hepatic necrosis and postnecrotic cirrhosis do not, (b) the serum tocopherol level tends to rise transiently immediately following acute hepatocellular injury or surgical manipulation of the bile duct, but does not fall below the control level in subsequent short-term periods of observation; similarly, there is no decrease in tocopherol concentration as a consequence of chronic carbon tetrachloride-induced cirrhosis, (c) the increase in serum tocopherol following an intramuscular injection of tocopheryl acetate is significantly greater in animals with biliary obstruction, bile fistula, or acute hepatic necrosis than in normal controls. This phenomenon is not due to the suppression of tocopherol excretion in the bile, nor is it, in the case of bile fistula and acute hepatic necrosis, related to the retention of cholesterol in the serum; however, the latter may be a factor in the case of biliary obstruction, since the rise in serum tocopherol concentration is significantly higher than in either of these other conditions.

These observations suggest that (a) acute hepatic injury, produced either by carbon tetrachloride administration or by surgical manipulation of the bile duct, leads to the release of tocopherol stored in the liver, and reduces the hepatic uptake of tocopherol from the serum, (b) complete interruption of bile flow into the intestinal tract impairs the absorption of tocopherol, (c) hepatocellular injury *per se* does not affect the serum concentration, absorption or tissue uptake of tocopherol, and (d) the factors leading to hypercholesteremia in obstructive jaundice may play a role in the retention of tocopherol in the serum.

The implications of these findings in the interpretation of alterations in tocopherol metabolism observed in human liver disease have been discussed.

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STUDIES OF THE SATIETY RESPONSE IN MICE[1]

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(Submitted for publication July 30, 1957; accepted August 15, 1957)

The increase in general activity which follows food deprivation in animals is well known (1). Less well known is the marked decrease in spontaneous running activity which occurs after a period of refeeding. Working with rats, Finger (2) demonstrated that after a period of food deprivation, feeding produced a marked decrease in spontaneous running activity as measured by the revolving cage technique. He called this response a "satiation syndrome." Studies in this laboratory (3) have confirmed this satiety response in mice. The present study was designed to determine some of the factors involved in this response.

METHODS AND MATERIALS

Female mice of the DBA strain obtained from the Jackson Memorial Laboratories, Bar Harbor, Maine, were used in this study. These mice were housed at constant temperature (72 F.), relative humidity (48 per cent), and with 12 hours of light each day. Mice under active study were placed in revolving activity cages modified from those described by Richter and Wang (4). After their daily activity, weights and food intakes had become relatively stable, they were subjected to the various manipulations described in the section Procedures and Results. Water was allowed *ad libitum* at all times. Ground Purina Chow pellets were fed unless otherwise indicated.

PROCEDURES AND RESULTS

Since the spontaneous activity covers a wide range among individual mice, the average activity for each mouse during the six day control periods has been taken as 100 per cent activity for charting purposes. The percentage of this base line for the mice in each study has been averaged for each day. Thus, the activity data are charted in terms of changes in activity during each study.

Figure 1 illustrates the changes in spontaneous running activity which occurred when six mice were deprived of food for 48 hours. On the first and second day of refeeding, there was a marked decrease in spontaneous running activity associated with a food intake above the control period average.

Figure 2 represents a study in which nine mice were allowed to have only 0.6 gram of food in the 24 hour period following a one day period of food deprivation. This limited amount of food produced a significant decrease in spontaneous running activity.

A mouse with electrolytic lesions (5) in the ventromedial centers of the hypothalamus which produced hyperphagia and obesity was subjected to a similar study. It is apparent in Figure 3 that this animal exhibited no significant activity changes on food deprivation or refeeding.

Figure 4 illustrates the results of a study of 12 mice given 0.1 mg. of chlorpromazine and 12 mice given 5 γ of reserpine subcutaneously twice daily. While both of these drugs produced marked decreases in spontaneous running activity, they did not affect the type of response to food deprivation and refeeding. The effect of oophorectomy on the

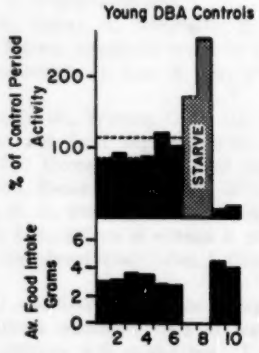

FIG. 1. DAILY PERCENTAGE OF CONTROL PERIOD ACTIVITY AND AVERAGE FOOD INTAKE FOR SIX NORMAL MICE DURING *Ad Libitum* FEEDING, STARVATION, AND REFEEDING

Broken lines indicate one standard deviation on either side of the control period average.

[1] This work was supported by a grant from the United States Public Health Service (A-893), and by Irwin, Neisler & Company.

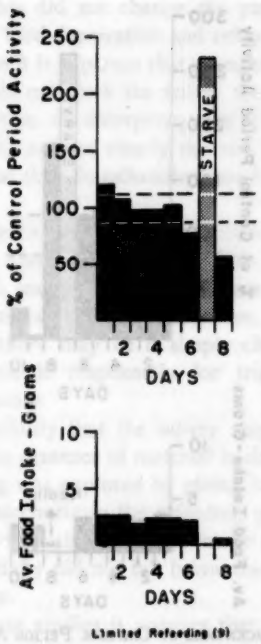

FIG. 2. DAILY PERCENTAGE OF CONTROL PERIOD ACTIVITY AND AVERAGE FOOD INTAKE FOR NINE MICE ALLOWED ACCESS TO ONLY 0.6 GM. OF FOOD THE DAY FOLLOWING A 24 HOUR PERIOD OF FOOD DEPRIVATION

Broken lines represent one standard deviation on either side of the control period average.

response to food deprivation and refeeding is given in Figure 5. Here oophorectomy produced the well known decrease in activity but it did not change the character of the response to starvation and refeeding.

The activity levels of nine mice allowed access to the protein lactalbumin, after 24 hours of fasting, are given in Figure 6. Although these mice ate an average of only 0.6 gram of this substance, a significant decrease in running activity occurred. Similar studies allowing only sucrose or lard after a 24 hour fast were done. An average intake of 3.0 grams of sucrose resulted in a decrease in running activity to 50 per cent of the control period activity while an intake of 1.6 grams of lard resulted in a decrease to 60 per cent.

Ten mice were offered kaolin sweetened with saccharin after a 24 hour fast. From Figure 7 it is evident that, though they ate an average of 0.8 gram of this substance, their running activity did not fall below the control period average.

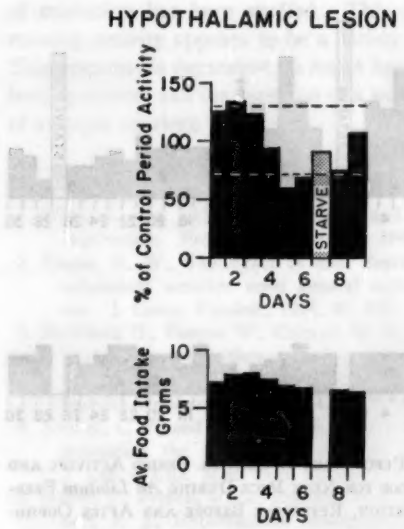

FIG. 3. DAILY PERCENTAGE OF CONTROL PERIOD ACTIVITY AND AVERAGE FOOD INTAKE IN A MOUSE WITH BILATERAL HYPOTHALAMIC LESIONS RESULTING IN HYPERPHAGIA AND OBESITY DURING *Ad Libitum* FEEDING, FOOD DEPRIVATION, AND REFEEDING

DISCUSSION

In planning these experiments, the question arose as to whether the satiety response was due to inanition or fatigue. From the study (Figure 1) in which mice were starved for 48 hours, it is apparent that the decrease in spontaneous running activity on refeeding after a 24 hour fast is

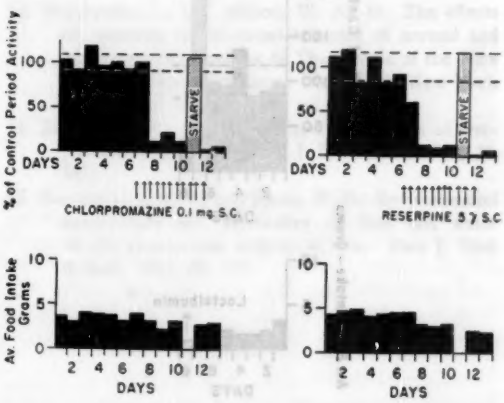

FIG. 4. DAILY PERCENTAGE OF CONTROL PERIOD ACTIVITY AND AVERAGE FOOD INTAKE OF 12 MICE GIVEN CHLORPROMAZINE AND RESERPINE DURING *Ad Libitum* FEEDING, STARVATION, AND REFEEDING

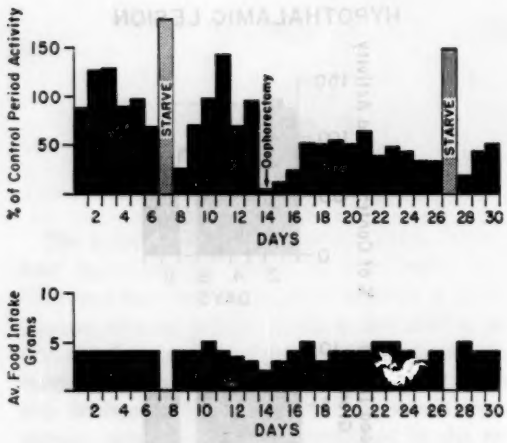

FIG. 5. PERCENTAGE OF CONTROL PERIOD ACTIVITY AND AVERAGE FOOD FOR NINE MICE DURING *Ad Libitum* FEEDING, STARVATION, REFEEDING BEFORE AND AFTER OOPHORECTOMY

not due to these factors. The observation that the mice consume more food than usual on refeeding after food deprivation (Figure 1) prompted the second study (Figure 2) in which mice were allowed access to a very limited amount of food in the refeeding period. This small amount was sufficient to produce a significant satiety response. Thus, the satiety response is not due to overeating.

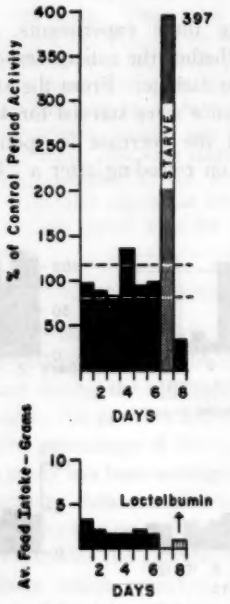

FIG. 6. PERCENTAGE OF CONTROL PERIOD ACTIVITY AND AVERAGE FOOD INTAKE FOR NINE MICE ALLOWED ACCESS TO *Ad Libitum* LACTALBUMIN AFTER 24 HOURS OF FOOD DEPRIVATION

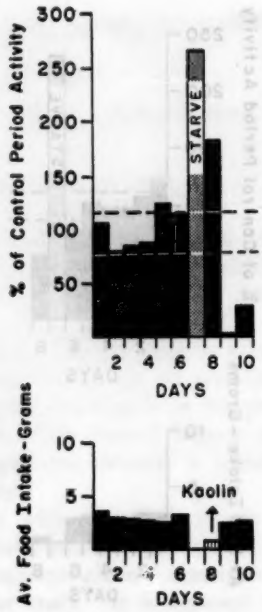

FIG. 7. PERCENTAGE OF CONTROL PERIOD ACTIVITY AND AVERAGE FOOD INTAKE FOR 10 MICE ALLOWED *Ad Libitum* KAOLIN SWEETENED WITH SACCHARIN AFTER A 24 HOUR FAST

The well established observations that rats (6, 7) and mice (5) with injuries in or around the ventromedian nuclei of the hypothalamus overeat and become obese were responsible for the study in Figure 3. Here a mouse with bilateral electrolytic lesions in the hypothalamus which resulted in hyperphagia and obesity was studied. In this study the satiety response did not occur nor did the usual increase in running activity on food deprivation. This, coupled with observations that mice with injury to the hypothalamic feeding centers produced by gold thioglucose (8) do not show changes in running activity on food deprivation or refeeding (3), indicates that intact hypothalamic feeding centers must be present for the satiety response to occur.

The mice given chlorpromazine and reserpine in Figure 4 showed a marked decrease in running activity on refeeding. Oophorectomy also produced a decrease in running activity (Figure 5);

however, this did not change the pattern of the response to food deprivation and refeeding. From these studies it is apparent that changes in the level of activity do not block the satiety response. The sites of action of chlorpromazine (9) and reserpine (10) are not clearly defined, but there is evidence that they do influence some hypothalamic centers.

The evidence that protein will produce a satiety response is given in Figure 6. This and similar studies with sucrose and lard indicate that simple nutrients produce a satiety response. This suggests that there may be a simple chemical substance which is responsible for triggering the satiety response.

The possibility that the satiety response might be due to the presence of material in the gut or the act of eating was explored by giving kaolin sweetened with saccharin in the refeeding period (Figure 7). Even with an average intake of 0.8 gram, running activity did not fall below the control period average.

From these studies it appears that the marked decrease in spontaneous running activity mice exhibit on refeeding after a period of starvation may be termed a satiety response. This response is dependent on intact hypothalamic feeding centers and the ingestion of at least a small amount of a simple nutrient. It is not affected by factors which change the general level of activity. This satiety response is not directly dependent on the volume ingested nor is it due to the act of eating or the presence of material in the gut. The role of the satiety response in the regulation of food intake is not clear. Studies of Brobeck (11) and Stevenson and Rixon (12) offer the idea that activity and food intake are related to the maintenance of body temperature.

SUMMARY

The marked decrease in spontaneous running activity mice exhibit on refeeding after a period of starvation has been studied. This change in running activity appears to be a satiety response. This response is dependent on intact hypothalamic feeding centers and the ingestion of a small amount of a simple nutrient.

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BLOOD ADRENOCORTICOTROPHIC HORMONE AND PLASMA CORTICOSTEROIDS IN MEN EXPOSED TO ADVERSE ENVIRONMENTAL CONDITIONS [1, 2]

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Electroshock (2), surgical trauma (3), accidental trauma and acute illness (4) are associated with elevations in plasma corticosteroids in man. It was of considerable interest to extend these observations to include adverse environmental conditions. Metabolic and hematologic changes similar to those resulting from the administration of adrenocorticotrophic hormone (ACTH) or corticosteroids have been reported to occur in human subjects experiencing hypoxia (5–7). In the present investigation blood ACTH and plasma corticosteroid concentrations were determined in subjects experiencing hypoxia, alone or in combination with heat. The influence of the adverse environmental conditions on the physiological well-being of the subjects was evaluated by measurement of changes in heart rate, respiration, body temperature, and urinary excretion of certain metabolites.

METHODS

Experiment 1. Short exposures; hypoxia induced by inhalation of a gas mixture low in oxygen. Seventy-nine healthy men, 18 to 35 years of age, served as subjects, each man serving on only one occasion. The subjects were divided into the following groups: (a) control, (b) hypoxia, (c) heat, and (d) hypoxia plus heat. During control and hypoxia tests, the environmental temperature was 27° C., relative humidity 50 per cent, and the subjects breathed by mask either room air or an oxygen-nitrogen mixture which, at ground level barometric pressure (750 mm. Hg), provided 100 mm. Hg inspired oxygen tension. Exposure to heat was accomplished in a cylindrical metal chamber in which air temperature (dry bulb) averaged 49° C., and the wall temperature 52° C., with relative humidity 18 to 20 per cent; under these conditions, one group breathed air and a second group the oxygen-nitrogen mixture.

After 2, 15 or 45 minutes of exposure, 20 ml. of blood were drawn from an antecubital vein into a heparinized syringe for corticosteroid analyses, then 200 ml. were drawn directly into glacial acetic acid for ACTH analysis. Plasma corticosteroids were determined by the method of Sweat (8). Blood was processed by the oxycellulose technique (9) and assayed for ACTH in hypophysectomized rats by the adrenal ascorbic acid depletion method. (10). A dose of eluate equivalent to 40 ml. blood was injected into each of five test rats. An estimate of the concentration of ACTH was based on the average response of the test rats to doses of unknown and the responses induced by the administration of 1.0 and 0.25 milliunits (mU) U.S.P. ACTH per 100 Gm. rat on the same day. As an additional control, five hypophysectomized rats were injected with 0.9 per cent NaCl solution in each assay.

Experiment 2. Three hour exposure; hypoxia induced by decompression in heated chamber. Five male subjects, 31 to 42 years of age, wearing shorts, were decompressed to the barometric pressure level equivalent to 14,000 feet altitude (total pressure, 446 mm. Hg; oxygen tension, 95 mm. Hg) in a chamber in which the air and wall temperatures were held at 51° C. and relative humidity at 13 per cent. Air was circulated through the chamber at a constant rate, and the subjects remained seated throughout the exposure. Cardiovascular, respiratory, and body temperature determinations were made periodically, and 20 ml. blood samples (venous) for corticosteroid, hematocrit, sodium, and potassium determinations were drawn immediately before the exposure, at the end of the second and third hours of exposure, and one hour after exposure. Urine samples collected one hour after exposure were analyzed by standard methods (11) for uric acid, creatinine, sodium, and potassium, and comparison was made with samples obtained at the same

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time on the previous day; on both occasions the urine samples represented a four hour accumulation.

Experiment 3. Two hour exposure; hypoxia induced by decompression in heated chamber. Eight male subjects, 18 to 23 years of age, were exposed in the same manner as in Experiment 2. Results are presented separately because the condition of the subjects made it necessary to terminate the exposure one hour earlier than had been intended. Rectal temperature, heart rate, and sweating rate determinations were made, and 20 ml. of blood were drawn just prior to exposure, at the end of two hours' exposure, and one hour postexposure.

RESULTS

Experiment 1

Blood samples from 54 of the 79 subjects were assayed for ACTH. Except for two subjects (one a control, and the other a heat-exposed subject), the ACTH titer was uniformly less than 0.5 mU per 100 ml. blood. The blood ACTH of the exceptional control subject was estimated to be 2 mU and that of the heat-exposed subject to be 0.5 mU per 100 ml. blood. No significant differences in the concentrations of 17-hydroxycorticosterone or of corticosterone-like steroids were found between the different groups of subjects. Data for 62 experimental subjects and for a group of 9 subjects who were not subjected to experimental procedures are summarized in Table I.

TABLE I

Plasma corticosteroids in relation to hypoxia and heat stress (experiment 1)

Treatment	No. of subjects	17-hydroxy-corticosterone (μg./100 ml. plasma)	Corticosterone-like steroids (μg./100 ml. plasma)
Control*	9	10.8 ± 0.8†	3.7 ± 0.4
Two-minute test			
Hypoxia	6	8.8 ± 1.3	4.2 ± 1.0
Fifteen-minute test			
Control	6	11.2 ± 0.4	4.8 ± 1.1
Heat	8	10.0 ± 0.8	5.8 ± 1.0
Hypoxia	6	13.3 ± 2.4	5.8 ± 1.5
Hypoxia plus heat	8	11.3 ± 1.8	5.6 ± 0.8
Forty-five minute test			
Control	6	10.4 ± 1.6	5.8 ± 1.8
Heat	8	11.7 ± 1.3	4.6 ± 0.8
Hypoxia	6	14.2 ± 3.2	5.1 ± 0.8
Hypoxia plus heat	8	12.6 ± 1.3	5.9 ± 0.3

* Nonexperimental subjects, 19 samples.
† Mean and standard error of the mean.

Changes in heart rate, systemic blood pressure, respiratory minute volume, and respiratory quotient tended to become maximal after approximately 15 minutes in the hypoxic subjects, whether at room temperature or in the hot chamber, and to remain fairly steady thereafter. The factor of heat had an intensifying effect on the response to hypoxia. To illustrate, heart rate, on the average, during hypoxia alone was not elevated to the same extent as with hypoxia plus heat, while hypoxia combined with heat induced an abrupt elevation in heart rate in contrast to a progressive increase with heat alone.

Experiment 2

The pre-exposure value for 17-hydroxycorticosterone in this group of five subjects was not significantly different from the control values obtained in Experiment 1 (Table II). The mean change noted at the end of two hours of exposure was not significant. All subjects exhibited increases in plasma concentration of 17-hydroxycorticosterone at three hours, although the increases for two of the subjects are of doubtful significance. When analyzed on the basis of the gain above the pre-exposure levels, the mean change was statistically significant ($> 0.01, < 0.02$). After a one hour recovery period, the average concentration had fallen to a level which was not significantly different from the pre-exposure level. Although showing the same tendency as 17-hydroxycorticosterone, the plasma concentration of corticosterone-like steroids was not significantly altered at any time. Hematocrit determinations did not indicate any significant degree of hemoconcentration.

As for the physiologic state, at the end of the exposure heart rates ranged from 105 and 125 beats per minute, oral temperatures from 37.4° to 38.0° C., mean skin temperatures (average of five equal areas) from 37.7° to 39.4° C., and ventilation (BTPS) from 4.7 to 27.6 liters per minute. Sweating rates (nude weight loss) ranged from 0.28 to 0.69 Kg. per M.^{2} per hour. One subject became incapacitated during the third hour, experiencing bradycardia, but he did not lose consciousness. Symptoms of hypoxia (cyanosis, euphoria, mental confusion, personality change), which were unusual for this altitude, were noted in all subjects. At the end of the one hour recovery period, heart rates and oral temperatures

TABLE II

Plasma corticosteroids in relation to decompression at high ambient temperature

	17-hydroxycorticosterone (μg./100 ml. plasma)	Corticosterone-like steroids (μg./100 ml. plasma)	Hematocrit (%)
Experiment 2 (5 subjects) *			
Pre-exposure	9.5 ± 0.9†	4.6 ± 0.9	43.8 ± 1.4
Two hrs. exposure	10.3 ± 2.0 (+0.8 ± 2.0)	5.0 ± 0.9	46.0 ± 1.2
Three hrs. exposure	15.3 ± 2.2 (+5.8 ± 1.4)	5.8 ± 0.6	45.0 ± 0.7
One hr. postexposure	11.1 ± 1.4 (+1.6 ± 1.6)	3.8 ± 0.3	44.8 ± 1.3
Experiment 3 (8 subjects) *			
Pre-exposure	10.2 ± 1.3	5.7 ± 1.2	45.8 ± 0.9
Two hrs. exposure	16.4 ± 3.4	7.8 ± 1.8	45.5 ± 1.0
One hr. postexposure	13.7 ± 2.8	7.2 ± 1.1	45.1 ± 0.8

* In Experiment 2 the barometric pressure is 446 mm. Hg; air and wall temperatures, 51° C.; relative humidity, 13 per cent. In Experiment 3 the barometric pressure is 446 mm. Hg; air temperature, 48° C.; wall temperature, 50° C.; relative humidity, 22 per cent.

† Mean and standard error of the mean; values in parentheses indicate mean increase above pre-exposure level along with standard error of the mean increase.

were not completely restored to the pre-exposure levels. Although there were no significant changes in plasma Na, K, or the Na/K ratio, changes in urine volume and in the output of certain urinary constituents clearly reflected the influence of the high temperature. Urine output on this occasion averaged 27 ml. per hour, whereas on the previous afternoon it had averaged 60 ml. per hour; the urinary uric acid/creatinine and Na/K ratios fell from 0.275 to 0.202 and from 1.55 to 0.55, respectively. The changes in these ratios were significant ($p < 0.02$ for the former, and $p < 0.01$ for the latter).

Experiment 3

The pre-exposure value for 17-hydroxycorticosterone for this group was not significantly different from control values in Experiments 1 and 2 (Table II). Although five of the eight subjects exhibited increases in plasma 17-hydroxycorticosterone levels after two hours of exposure, the average of the individual changes (+ 6.2 ± 2.8) was not significantly different from zero. Corticosterone-like steroids also were not changed significantly. In four of the eight cases, values for 17-hydroxycorticosterone were elevated after a one hour recovery period when compared with pre-exposure levels. Hematocrit values were not changed significantly.

Subjectively, this group of subjects found the exposure to be a distressing experience. Two subjects became incapacitated; in both cases heart rate was approaching 140 beats per minute, a rate which, for inactive subjects, is considered a critical limit. When ranked on the basis of an index of physiologic strain (12), which evaluates in quantitative terms the effects of the thermal environment, the subjects with the three highest strain values exhibited increases in 17-hydroxycorticosterone in the range + 5.2 to + 18.9 μg. per 100 ml. plasma. Of the remaining subjects, two were unable to remain in the chamber for two hours, and the strain index was not computed; the other three had the lowest strain values, and for these subjects the changes in the steroid concentration were in the range − 1.0 to + 6.1 μg.

DISCUSSION

The results confirm previous observations (13) that blood of normal male subjects contains less than 0.5 mU ACTH per 100 ml., and the observations of Paris, Upson, Sprague, Salassa, and Albert (14), who have been unable to detect ACTH in the blood of normal subjects. The estimate is based on the fact that 0.25 mU ACTH induces a response in test rats, and the fact that the equivalent of 40 ml. of blood fails to induce a significant depletion of adrenal ascorbic acid. The relatively high recovery of ACTH by the oxycellulose method (10) indicates that failure to

detect the hormone in blood is not due to loss incurred during processing.

It is to be emphasized that our failure to detect ACTH in the blood of subjects exposed to hypoxia does not rule out increased rate of release of ACTH from the adenohypophysis. Of interest in this connection are the observations of Thorn, Jenkins, and Laidlaw (7) that a very small dose of ACTH given by constant intravenous infusion (0.25 unit per hour) induces significant increases in urinary excretion of corticosteroid metabolites. This rate of infusion of ACTH would not be expected to increase blood ACTH to a level detectable by our method. More sensitive methods for the analysis of blood ACTH must be developed before we can clarify the problem of ACTH release in response to noxious stimuli in man.

Franksson and Gemzell (15) have presented data which they interpret to mean that the apprehension immediately prior to surgery is associated with an increase in peripheral plasma corticosteroid levels as measured by the method of Nelson and Samuels (16). In the present experiments subjects were noticeably apprehensive, yet the pre-exposure levels did not differ from those of untreated controls.

The fact that the plasma corticosteroids did not increase in concentration during the first 45 minutes of exposure to hypoxia cannot be explained on the basis of latency or inertia of the pituitary-adrenocortical system. ACTH can be discharged at an accelerated rate within two minutes after application of stress to experimental animals (17), and the peripheral blood steroid level of man exhibits an increase within 15 minutes after the start of an intravenous infusion of ACTH (18). A significant increase in plasma corticosteroids has been reported to occur within 15 minutes after electroshock seizure in man (2).

We cannot conclude with certainty that the secretory activity of the adrenal cortex was increased during the longer exposures; we are only justified in saying that an imbalance between production and elimination resulted in an increase in the concentration of 17-hydoxycorticosterone in the plasma after two hours of exposure to adverse environmental conditions. Tyler, Schmidt, Eik-Nes, Brown, and Samuels (19) have presented evidence which suggests that reduction in rate of steroid degradation by the liver is in part responsible for the increase in peripheral blood steroid levels which occurs during and immediately following surgery. Regardless of the cause, increased rate of secretion or decreased rate of elimination, it is of some importance to find that certain individuals respond to heat plus hypoxia with an increased concentration of 17-hydroxycorticosterone in the plasma. Whether an increase of plasma corticosteroids above "nonstress" levels is of value to the individual in resisting the effects of heat plus hypoxia remains unanswered.

SUMMARY

Hypoxia (equivalent to that at 14,000 feet altitude), high environmental temperature (50° C.), or hypoxia in combination with heat induced no detectable increase in the titer of ACTH in peripheral blood or in the concentration of plasma 17-hydroxycorticosterone, or corticosterone-like steroids of healthy male subjects exposed for brief periods (not in excess of 45 minutes). In a second experiment, three of five subjects who experienced hypoxia of this degree in a heated decompression chamber exhibited significant increases in the concentration of peripheral plasma 17-hydroxycorticosterone after three hours of exposure. In a third experiment, five of eight subjects exhibited increases in plasma concentration of corticosteroid after two hours of exposure.

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TOXICITY AND BLOOD AMMONIA RISE RESULTING FROM INTRAVENOUS AMINO ACID ADMINISTRATION IN MAN: THE PROTECTIVE EFFECT OF L-ARGININE

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A study of amino acid toxicity was undertaken following the observation that a mixture of intravenously administered L-amino acids that had been nutritionally adequate in a patient became toxic when L-arginine was deleted. This toxicity was unexpected for the amino acid mixture, even without arginine, contained all of the amino acids determined by Rose (1) to be essential to man. Readdition of L-arginine, however, prevented the toxicity. Subsequent studies by Guillino, Winitz, Birnbaum, Otey, Cornfield, and Greenstein suggested that the toxicity of arginine deficient L-amino acid mixtures might be attributable to a blood ammonia rise (2, 3).

Investigation of the arginine-deficient amino acid mixture in dogs (4) demonstrated a marked blood ammonia rise on intravenous administration and indicated that the toxic manifestations, convulsion and death were attributable to ammonia formation. L-arginine administration prevented the blood ammonia rise and toxicity.

The present studies were undertaken to demonstrate in man the blood ammonia rise that can develop from intravenous administration of an L-amino acid mixture deficient in arginine, and to confirm the capacity of L-arginine to significantly reduce the blood ammonia changes produced by this mixture. Glycine was also used to produce an elevation of blood ammonia, and the role of L-arginine in preventing this rise was investigated.

MATERIALS AND METHODS

Solutions of amino acids containing all of the amino acids "essential" for man (1) were prepared so that each 250 ml. contained at least the minimal amount of all essential amino acids required for daily human maintenance (see Table I).[1] L-alanine was included to provide nitrogen for synthesis of the nutritionally nonessential amino acids.

Individual 1.0 molar solutions of L-arginine·HCl and L-ornithine·HCl, and 1.7 molar solutions of glycine were prepared with pyrogen-free distilled water and sterilized in the autoclave.[2] All solutions were tested by routine methods in the Division of Biologics Standards, National Institutes of Health, and found to be free of pyrogen and bacterial contaminants. Final concentration was checked by the Kjeldahl nitrogen determination.

Ammonia determination. Blood ammonia was determined on duplicate samples by a modification of the Seligson microdiffusion method (6, 7). Two and a half ml. of blood are collected in a heparinized syringe, transferred to a clean test tube, and one ml. pipetted immediately into a 60 ml. wide-mouth serum bottle (U.S. Army Medical Corps Pattern) to which had previously been added 2 ml. of a saturated potassium carbonate solution. An etched glass rod mounted in a rubber stopper is dipped into 2 N sulfuric acid and promptly inserted into the bottle mouth. This entire operation, from appearance of blood in the syringe to stoppering of the bottle, requires about one minute. A blank preparation without blood is prepared at the same time. The bottles are then mounted on a 16 inch diameter diffusion wheel and rotated at 37 r.p.m. for 20 minutes. At the end of this time the glass rod is removed from the bottle and inserted into a Coleman 10 by 75 mm. cuvette containing 3 ml. of a 1:10 dilution of Nessler's Reagent, inverted five times and removed, allowing drainage of the reagent from the rod. After 10 minutes at room temperature, the optical density at 420 millimicrons is read after setting the blank at zero density. With one microgram of ammonium nitrogen (NH_3-N) the optical density was in the range of 0.065. Our experience is in agreement with that of Seligson (6, 7) with complete recovery of ammonia from ammonium sulfate standard solutions and 90 to 100 per cent recovery of ammonia added to blood. Each day a control curve was obtained by carrying out the entire procedure on samples of ammonium sulfate containing 1, 2, 5 and 10 micrograms of NH_3-N. This curve is a straight line to 5 and tapers slightly to 10 micrograms of NH_3-N.

The mean fasting venous blood ammonia value of 80 determinations in seven individuals with localized neoplasms but without any evidence of liver disease fol-

[1] L-amino acids prepared by enzymic resolution (5) were generously supplied by members of the staff of the National Cancer Institute. Glycine was obtained from the Nutritional Biochemical Company, Cleveland, Ohio, and a portion of the L-arginine·HCl was obtained from the Mann Research Laboratories, Inc., New York, N. Y.

[2] These solutions were prepared with the assistance of Dr. J. A. Scigliano of the Pharmacy Department, Clinical Center, National Institutes of Health.

TABLE I

Intravenous amino acid mixtures

Amino Acid Composition	Solutions B-1 (gms/L†)	B-2 (gms/L†)
L-Tryptophane	1.0	1.0
L-Phenylalanine	4.4	4.4
L-Lysine	3.4	3.4
L-Threonine	2.0	2.0
L-Valine	3.2	3.2
L-Methionine	4.4	4.4
L-Leucine	4.4	4.4
L-Isoleucine	2.8	2.8
L-Arginine	4.4	0
L-Histidine	4.4	0
L-Alanine	112.8	120.4

lowed for one to nine months with 4 to 23 determinations per patient was 1.1 ± 0.2 (S.D.) micrograms of NH_3-N per ml. The one subject who was followed for nine months with 23 determinations had a mean venous blood ammonia value of 1.2 ± 0.2 (S.D.) micrograms of NH_3-N per ml. Similarly, single observations in 20 normal individuals averaged 1.2 ± 0.2 (S.D.) mg. NH_3-N per ml.

Amino acid infusions. The arginine-free L-amino acid mixture was administered to four fasting subjects (M.M., J.F., W.P., D.G.) who had received their usual morning and noon diet, but the evening intake was free of protein on the day prior to study. Breakfast was omitted on the day of study. Glycine was infused into four subjects (G.S., W.P., J.F., G.A.) who had been fasted from protein while receiving adequate water, electrolytes and 300 grams glucose during the 36 hours prior to each study. The infusion studies generally were separated by a one week interval. One subject (W.M.) received glycine after dietary preparation as outlined above for the amino acid mixture.

The 60 minute infusions were administered through a Bowman constant infusion pump into a peripheral vein. Serial blood samples for ammonia determinations collected through a Cournand needle placed in an antecubital vein were obtained before, during, and after amino acid administration. Ammonia determinations were carried out immediately upon obtaining the blood. L-arginine was injected intravenously and separately from the amino acid infusion, unless otherwise noted. A total of 53 studies was carried out in seven subjects.

Subjects. All of the subjects who received amino acids intravenously presented significant problems in the maintenance of nutrition. All but one required either complete parenteral alimentation or cervical esophagastomy and intragastric tube feedings.

M.M., a 63 year old woman, had epidermoid carcinoma of the face. J.F. and G.S., respectively 56 and 55 year old men, had carcinoma of the buccal mucosa. W.P., a 42 year old man, and D.G., a 55 year old woman, had carcinoma of the stomach. G.A., a 59 year old man, had recurrent esophageal carcinoma, a liver palpable 3 cm. below the right costal margin and normal liver function tests except for elevated alkaline phosphatase levels (4.5 to 9.2 Bodansky units), although at autopsy the 1,800 gram liver had no evidence of metastatic neoplasm. W.M., a 57 year old man with biopsy proven hepatoma that was symptomatic only through a painful mandibular metastasis, was found to have abnormal bromsulphalein retention (20 per cent), a 4 plus cephalin flocculation test, thymol turbidity of 5 units, and an elevated serum globulin level, although the serum albumin, alkaline phosphatase and prothrombin time were normal. This patient is alive without evidence of hepatic failure eight months after these studies were performed.

Although all of the subjects had a malignant disease, none had evidence of renal or hematologic abnormality. Only W.P. and G.A. had lost weight prior to these observations, and only W.M. and G.A. had evidence of hepatic disease. Because these patients required intragastric tube feedings or entirely parenteral alimentation (except W.M.), dietary control was easily achieved. None lost weight during the period of experimental observation. None had edema, ascites or other evidence of fluid retention.

RESULTS

Initial observation of amino acid toxicity

In the course of observations on the nutritional value of amino acid mixtures administered intravenously as the sole source of nitrogen, a mixture of eight "essential" L-amino acids plus L-histidine, L-arginine and L-alanine was found to be non-toxic and nutritionally adequate in the patient who received this solution (Solution B-1, Table I). Positive nitrogen balance is demonstrated in Figure 1.

Another intravenous amino acid mixture (Solution B-2), essentially the same except for deletion of L-arginine and L-histidine, induced coma in this same patient. This 45 Kg. man, totally dependent on intravenous feeding because of multiple intestinal obstructions secondary to gastric carcinoma, during a period of six hours received 250 ml. of this solution, which was sterile and pyrogen free. Two hours after the end of the infusion he was noted to be unresponsive. Neurologic examination revealed generalized rigidity, plastic resistance to passive movements, and bilateral Babinski responses. No definite localizing signs developed. Blood sugar and calcium levels obtained during coma were normal. Within 24 hours the patient became responsive to painful and auditory stimuli, and in another two days was entirely oriented and demonstrated normal motor function and reflexes. During this period of recovery, the

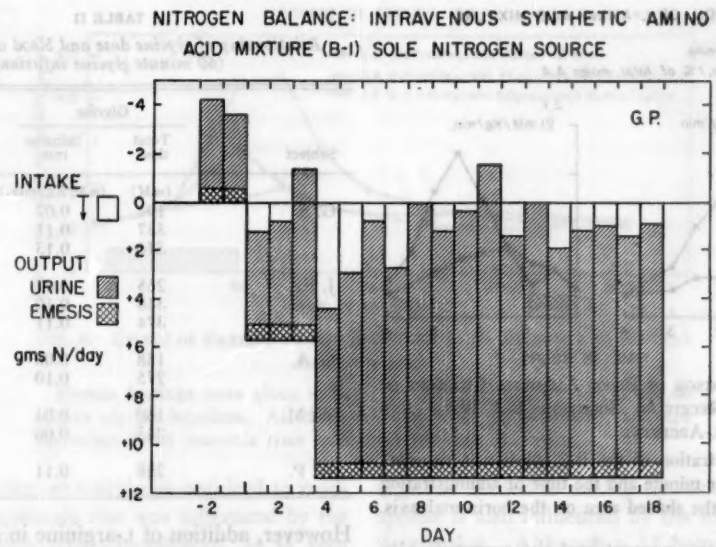

FIG. 1. POSITIVE NITROGEN BALANCE IN A SUBJECT RECEIVING L-AMINO ACID MIXTURE B-1 AS SOLE SOURCE OF NITROGEN

amino acid mixture containing L-arginine (B-1) was administered without evidence of toxicity.[3]

[3] Because this episode differed somewhat from the toxic manifestations usually seen with transitory blood ammonia elevations, additional information may be of interest. Prior to this episode, there was no evidence of hepatic, renal or central nervous system disease. However, this patient had undergone a 24 Kg. weight loss in the previous two years, had been on a limited diet necessitated by the requirement for intravenous feeding during the preceding four months, and was in a state of mild folic acid deficiency (M.C.V., 105 cubic microns; hematocrit, 31 per cent; B_{12} intake, adequate; marked [12 per cent] reticulocyte response to subsequent folic acid administration). Nausea and retching were noted in the two hours prior to the development of coma but similar symptoms had been present for several months and were attributed to the almost complete obstruction of the intestinal tract. This patient, when first seen in coma, had a respiratory rate of four per minute and had received four hours earlier 20 mg. of morphine sulfate, which represented a moderate increase in his regular narcotic medication. Because of the possibility of morphine poisoning, 10 mg. of allomorphine were given. Later, trembling movements of the head and extremities consistent with acute narcotic withdrawal became evident and gradually subsided over 30 hours. No tonic or clonic convulsions were observed. Fever developed 10 hours after the coma was first noted and persisted (daily peaks of 38.6 to 39.6° C.) for five days, during which penicillin and tetracycline were given. Although a leukocytosis as high as 30,000 w.b.c. per cu. mm. was present during the first two days of fever and returned to normal two days later, clinical, bacteriologic and roentgenographic studies revealed no evidence of an infection. During the 10 days following the coma episode, the nitrogen balance averaged −3.0 grams per day in contrast to −0.4 gram prior to this episode. The daily intake was 5.0 grams nitrogen and 1,200 calories in both periods. Other febrile episodes in this patient were not associated with such markedly negative nitrogen balances. At autopsy examination three months later, no organic brain disease was evident which might have accounted for this episode.

L-amino acid solution: Ammonia production and arginine protection

After extensive investigation in experimental animals of the changes associated with intravenous administration of the arginine deficient L-amino acid mixture (4), this solution was administered intravenously to subjects M.M. and J.F. with a resultant rise in blood ammonia (Figure 2). Addition of L-arginine to the infusion mixture strikingly inhibited the ammonia rise. Quantities of L-arginine (6 and 7 millimoles) corresponding to only 1 per cent of the total amount of amino acids infused were effective. Observations in subject D.G. also revealed the same effect of L-arginine on the potential blood ammonia rise (Figure 3). Nausea and, rarely, retching were observed in association with blood ammonia over levels 6 μg. NH_3-N per ml., but were definitely diminished

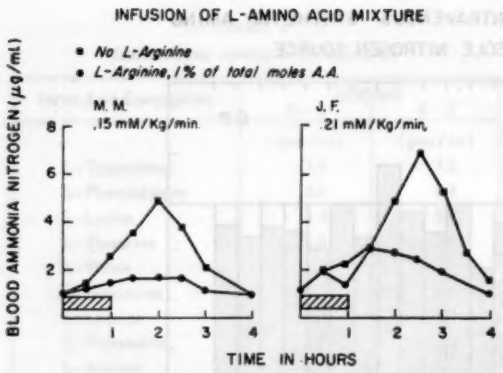

FIG. 2. COMPARISON OF BLOOD AMMONIA CHANGES IN TWO SUBJECTS RECEIVING SOLUTION B-2 WITH AND WITHOUT ADDED L-ARGININE

Rate of administration of the B-2 solution is recorded in mM per Kg. per minute and the time of administration is represented by the shaded area on the horizontal axis.

when arginine was present and the ammonia rise was reduced. No other toxic sign or symptom was noted.

These studies demonstrate the ammonia-forming potential of the toxic Solution B-2, an L-amino acid mixture that does not contain L-arginine.

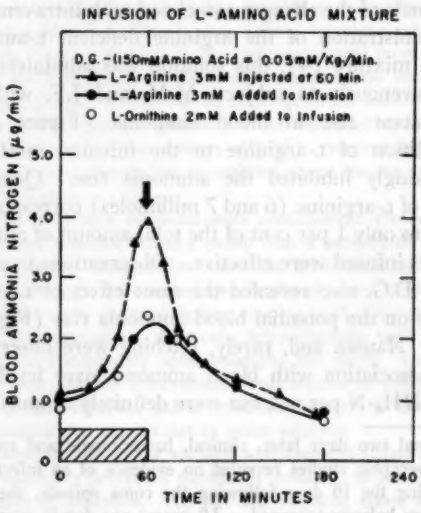
INFUSION OF L-AMINO ACID MIXTURE
D.G. - (150mM Amino Acid = 0.05mM/Kg/Min.
L-Arginine 3mM Injected at 60 Min.
L-Arginine 3mM Added to Infusion
L-Ornithine 2mM Added to Infusion
BLOOD AMMONIA NITROGEN (μg/ml.)
TIME IN MINUTES

FIG. 3. EFFECT OF L-ARGININE AND L-ORNITHINE ON THE BLOOD AMMONIA CHANGES ASSOCIATED WITH INFUSION OF ARGININE-DEFICIENT L-AMINO ACID MIXTURE B-2

The shaded area indicates the time of amino acid (B-2) infusion. In one study the B-2 mixture was infused alone and arginine was injected intravenously, indicated by the arrow (↓), immediately afterward.

TABLE II

Relationship of glycine dose and blood ammonia rise (60 minute glycine infusion)

Subject	Glycine: Total dose	Glycine: Infusion rate	Venous blood: Ammonia rise
	(mM)	(mM/Kg./min.)	(μg./ml.)
G. S.	194	0.07	3.3
	337	0.11	6.5
	376	0.13	7.8
J. F.	265	0.07	1.1
	348	0.10	2.1
	374	0.11	3.1
G. A.	158	0.06	0.4
	275	0.10	2.2
W. M.	160	0.04	0.4
	245	0.06	4.3
W. P.	288	0.11	1.0

However, addition of L-arginine in small amounts prevented or markedly reduced the blood ammonia changes. It should be noted that the similar but non-toxic Solution B-1 contains somewhat more L-arginine than was necessary to modify the ammonia-forming potential of mixture B-2.

Glycine-ammonia production

In further studies of ammonia production from intravenous amino acid infusion glycine was utilized. The influence of the rate of glycine administration on the blood ammonia rise is indicated in Table II. With increasing rates of glycine administration, a greater blood ammonia response was noted. Because all infusions lasted 60 minutes, a comparison of the effect of total dose versus rate of glycine administration cannot be made.

Some degree of fasting in the amino acid recipient is usually required to produce a blood ammonia rise on amino acid administration, at least under the conditions in which these studies were conducted. A comparison of the blood ammonia response at several levels of protein feeding is illustrated in Figure 4.

Protective effect of L-arginine

Intravenous administration of L-arginine immediately prior to glycine infusion was capable of preventing the ammonia rise in fasted subjects (Figure 5). A partial inhibition of the ammonia rise was produced by suboptimal amounts of argi-

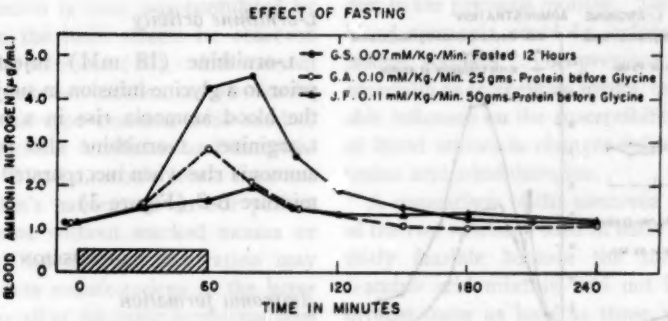

FIG. 4. EFFECT OF FASTING ON THE BLOOD AMMONIA RESPONSE TO GLYCINE ADMINISTRATION

Protein feedings were given to G.A. and J.F. two hours prior to the 60 minute glycine infusions. All three subjects, when fasted equally, had had equivalent blood ammonia rises with these amounts of glycine.

nine. The amount of L-arginine required to modify the blood ammonia rise was influenced by the amount of amino acid administered, as is seen with subject G.S., Figure 5. When an increased amount of glycine was given, more arginine was needed to reduce the blood ammonia changes. The characteristics of the amino acids administered may also influence the arginine requirement. This is suggested by the apparently lower arginine requirement of the L-amino acid mixture than of the single amino acid glycine. The quantity of arginine needed to modify the blood ammonia response is also influenced by the extent of protein deprivation. Although a 12 hour fast is usually adequate to produce a deficiency state demonstrable under the conditions of these experiments, a 36 hour period without protein intake was routinely employed in these studies.

The rapidity and effectiveness of L-arginine in altering a blood ammonia rise that is already underway were investigated by withholding the L-arginine injection until the forty-fifth minute of a 60

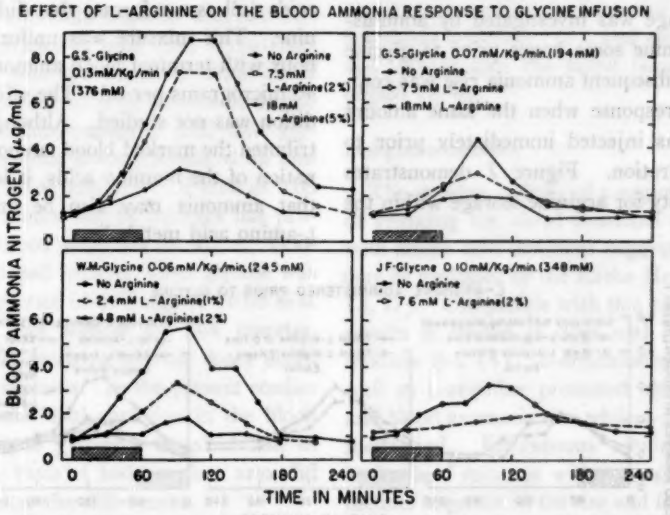

FIG. 5. EFFECT OF L-ARGININE ON THE BLOOD AMMONIA RESPONSE TO GLYCINE INFUSION

L-arginine was injected intravenously immediately prior to the glycine infusion.

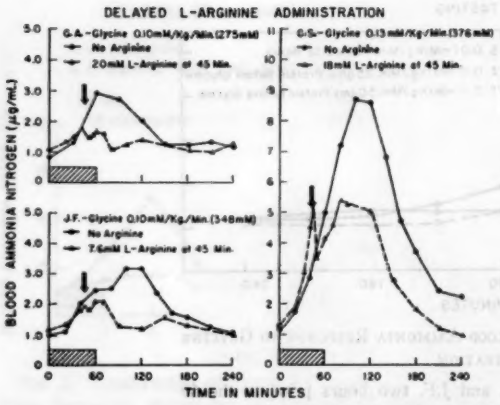

FIG. 6. EFFECT OF DELAYED L-ARGININE INJECTION ON THE BLOOD AMMONIA RISE ALREADY UNDERWAY FROM GLYCINE ADMINISTRATION

The shaded area indicates the duration of glycine infusion. The time of arginine injection is indicated by the arrow (↓).

minute glycine infusion. As is seen in Figure 6 (and Figure 3), the L-arginine injection was capable of preventing further blood ammonia rise and inducing an early return to normal levels. The effectiveness of the L-arginine injection evidently depends upon the quantity of L-arginine injected and the amount of amino acid infused.

Arginine storage was investigated by administration of L-arginine some hours prior to glycine infusion. The subsequent ammonia rise was compared with the response when the same amount of L-arginine was injected immediately prior to glycine administration. Figure 7 demonstrates an evident capacity for arginine storage within the body.

L-ornithine activity

L-ornithine (18 mM) injected intravenously prior to a glycine infusion in subject G.S. inhibited the blood ammonia rise in a manner similar to L-arginine. L-ornithine also reduced the blood ammonia rise when incorporated in the amino acid mixture B-2 (Figure 3).

DISCUSSION

Ammonia formation

Blood ammonia elevation in association with amino acid administration has been demonstrated previously in animal studies. Ammonia release into the blood of dogs following glycine administration has been reported by Bornstein (8), Polonovski, Boulanger, and Bizard (9), Doolan, Harper, Hutchin, and Alpen (10), and Harper, Najarian, and Silen (11). Greenstein and coworkers, investigating the toxicity of mixtures and individual L-amino acids administered intraperitoneally to rats, demonstrated that a marked blood ammonia rise may accompany the toxic manifestations of L-amino acid administration and suggested that the ammonia formation was one aspect of L-amino acid toxicity (12, 13). Kamin and Handler (14), administering amino acids parenterally to dogs, infused a mixture of amino acids (all as D-L forms) that did not contain arginine. This mixture was uniformly lethal in one hour with terminal blood ammonia levels of 30 to 40 micrograms per ml. The effect of arginine addition was not studied. Although the authors attributed the marked blood ammonia rise to deamination of the D-amino acids, it is now appreciated that ammonia may also be an intermediate in L-amino acid metabolism.

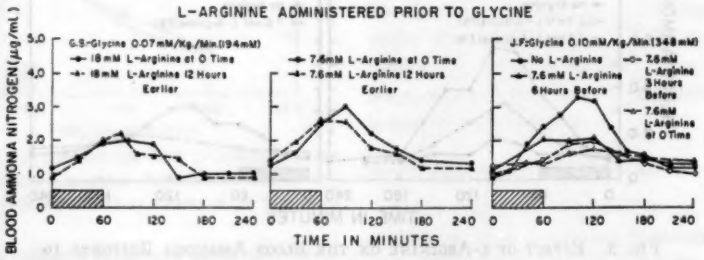
L-ARGININE ADMINISTERED PRIOR TO GLYCINE
G.S.-Glycine 0.07mM/Kg/Min.(194mM)
18mM L-Arginine at 0 Time
18mM L-Arginine 12 Hours Earlier
7.6mM L-Arginine at 0 Time
7.6mM L-Arginine 12 Hours Earlier
J.F.-Glycine 0.10mM/Kg/Min.(348mM)
No L-Arginine
7.6mM L-Arginine 6 Hours Before
7.6mM L-Arginine 3 Hours Before
7.6mM L-Arginine at 0 Time
BLOOD AMMONIA NITROGEN (μg/mL)
TIME IN MINUTES

FIG. 7. COMPARISON OF THE BLOOD AMMONIA RESPONSE TO GLYCINE INFUSION WHEN L-ARGININE WAS INJECTED AT VARIOUS INTERVALS PRIOR TO GLYCINE ADMINISTRATION

Ammonia formation in man was postulated by Doolan to explain the toxic effects he observed with rapid glycine infusion (10). His normal fasting subject experienced malaise, nausea, vomiting and a pyrogenic-type reaction with marked weakness, shaking chills, headaches and temperature to 100.8° F. Blood ammonia levels were not measured in Doolan's patient nor in our patient who developed coma without marked nausea or vomiting, although narcotic administration may have suppressed these manifestations in the latter instance. Whether all of the toxic symptoms seen in the patient receiving the amino acid mixture B-2 are attributable to ammonia toxicity and whether advanced debility contributed to the response is uncertain. In our studies with controlled blood ammonia elevation from administration of glycine or an L-amino acid mixture, nausea and retching accompanied the blood ammonia rise. No gross neurologic or cardiovascular changes were noted. Nausea has also been noted when blood ammonia elevation was produced by ammonium chloride infusion (15). While other metabolic products from amino acid administration may be toxic, ammonia formation with resulting elevated blood ammonia levels is one aspect of amino acid toxicity.

The amount of amino acid administered during the studies reported here was found to determine, in part, the blood ammonia response and toxicity. The relative importance of total dose versus rate of administration was not assessed since all of the studies were conducted with 60 minute amino acid infusions. Administration of 0.10 mM glycine per Kg. per minute always produced an ammonia rise in our fasted subjects. As little as 0.06 mM per Kg. per minute for 60 minutes has produced significant blood ammonia elevation. Doolan's subject developed toxicity when glycine was given at an average rate of 0.67 mg. N (0.05 mM glycine) per Kg. per minute for 104 minutes. Two other subjects receiving smaller doses noted only mild toxic symptoms. In the present studies there was some individual variation in the blood ammonia response to glycine. Recalculation of the dosage on the basis of body surface area did not significantly alter the differences.

It should be noted that the patient in whom the toxic effects of the arginine-free amino acid mixture were first observed had been on a low-arginine diet in the previous months. Similarly, the rise in blood ammonia could be induced most easily in fasting subjects. The previous nutritional state, especially as to arginine intake, may have considerable influence on the susceptibility of development of blood ammonia changes following intravenous amino acid administration.

A comparison of the ammonia forming potential of the two solutions used in these studies is not entirely feasible because the subjects given the L-amino acid mixture had not been fasted from protein quite as long as those receiving glycine. However, different rates of ammonia formation are indicated if the sequence of ammonia changes are compared when equivalent blood ammonia elevations were induced by the two solutions. With glycine the 60 minute blood ammonia level is higher and the maximum rise is obtained earlier than with the B-2 mixture which is composed principally of L-alanine. This is compatible with the observations in the rat where individual L-amino acids produced characteristic and differing blood ammonia responses after intraperitoneal injection (12). The relative contribution of the infused amino acid nitrogen to the NH_3-N that actually reaches the blood is uncertain. However, it is not unreasonable to assume that some ammonia comes from the administered amino acids. In experimental animals, both glycine and L-alanine are rapidly removed by the liver and kidneys after intravenous injection (16), and ammonia formation and release into the blood occur in both these organs (8, 9).

L-arginine activity

The striking activity of L-arginine in preventing or reducing the blood ammonia rise and toxicity with amino acid infusions suggests that this effect may be mediated by the Krebs-Henseleit urea cycle (3, 17). Compatible with this explanation are the results of studies in dogs with the L-amino acid mixture B-2 (4). L-ornithine and L-citrulline as well as L-arginine protected against the toxicity and blood ammonia rise while L-glutamic acid was ineffectual. Intravenous administration of the amino acid mixture without arginine produced a distinct sequence in the rise and fall of amino acids, ammonia, and urea in the blood with peak levels being reached in that order. Such changes are compatible with a sequence of amino acid removal from

the blood and metabolism to produce ammonia, ammonia production temporarily exceeding the capacity for ammonia removal and, finally, cumulative urea synthesis resulting in an increased blood urea. By measuring both the urine urea output and plasma urea rise, it was possible to demonstrate that urea production was more rapid when L-arginine was included in the amino acid mixture and that the increased urea was more than would be contributed by direct metabolism of the administered arginine. Similar effects of L-arginine on the blood ammonia and urea concentrations have been demonstrated following administration of L-amino acids to rats (13) and glycine to dogs (18).

L-arginine was effective in man when administered by intravenous rapid injection or slow infusion. The protective action of dietary protein suggests that orally administered arginine would also be active. L-arginine was active if included in the amino acid infusion or if injected shortly before. There is some evidence that administration of small amounts of arginine may be less effective if given more than three to six hours prior to the ammonia-inducing amino acids. This has been demonstrated somewhat more clearly in dogs (4) than in the present studies in man. If the amino acid infusion and blood ammonia rise are already underway, L-arginine administration apparently acts rapidly to modify the extent and duration of the ammonia rise.

The amount of L-arginine needed to modify significantly the blood ammonia changes resulting from amino acid infusion in man will vary with the amount, rate and ammonia-forming potential of the administered amino acid(s) and with the nutritional state of the host. In these studies with glycine and with a mixture of L-amino acids, 5 to 10 millimoles (approximately 1 to 2 grams) of L-arginine effectively reduced blood ammonia changes when the tendency to ammonia formation was not great. However, when the blood ammonia change was likely to be large, as much as 20 millimoles (4 grams) of L-arginine was only partially effective.

No toxicity was observed with L-arginine·HCl administration either as a 20 mM intravenous injection in three minutes or as an 180 mM (38 grams) infusion over 120 minutes. L-ornithine·HCl was non-toxic when given as a 20 mM injection.

The rates of amino acid administration in these studies do not markedly differ from those of clinical intravenous alimentation. Infusion in an hour of a liter of typical 5 per cent protein hydrolysate (containing 6 grams of nitrogen with 4.2 grams as alpha amino nitrogen) to a 50 Kg. patient represents a rate of 0.10 mM amino acid per Kg. per minute and is in the range utilized in these studies. Giving a fairly complete amino acid mixture may have markedly different effects from those associated with an unbalanced mixture or a single amino acid. The major effect observed in these studies seems to be related to the presence or absence of L-arginine.

The presence of neoplasm in these subjects is not thought to alter the general application of these findings to man. The extent that hepatic disease will affect the response to amino acid administration is uncertain. In our patient with conspicuous hepatic disease (W.M.), the ammonia rise developed readily with glycine administration, and L-arginine was effective in reducing this rise. Kirk did not observe any marked difference from normal in the metabolism of orally administered glycine in patients with hepatic disease (19).

Intravenous amino acid administration has been utilized as a liver function test (20–22) and in renal tubular function studies (10), and intravenous glycine has been proposed as a means of combating hypothermia (23). Purified amino acids given intravenously have been utilized in physiologic and nutritional studies in man. Such situations emphasize the need for recognition of the role of L-arginine. Although L-arginine may not be "essential" to the maintenance of nitrogen balance in healthy young adults fed an oral diet, it would seem to be important to regard L-arginine (or a related amino acid) as "essential" in the prevention of one aspect of intravenous amino acid toxicity, that of excessive ammonia in the blood.

CONCLUSIONS

1. Marked blood ammonia rise and toxicity have been demonstrated in man with intravenous administration of glycine or an L-amino acid mixture free of L-arginine but complete in all the "essential" amino acids.

2. The blood ammonia rise with intravenous amino acid administration appears to depend on the amount of amino acid and rate at which it is given, the ammonia-forming characteristics of the

amino acid(s) infused, and the state of nutrition of the host, particularly in relation to recent arginine intake.

3. L-arginine was effective in reducing the blood ammonia rise and associated toxicity developing from these intravenous amino acid infusions. L-arginine may be given as a part of the infusion or by intravenous injection immediately or some hours prior to the amino acid administration. If amino acid administration and the blood ammonia rise are already underway, injection of adequate amounts of L-arginine will rapidly reduce the blood ammonia rise.

4. The findings indicate that a functioning metabolic pathway, requiring L-arginine and consistent with the operation of the Krebs-Henseleit cycle, is an important metabolic route when large amounts of amino acids are administered to man.

ACKNOWLEDGMENT

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EFFECTS OF POSTURE AND ATROPINE ON THE CARDIAC OUTPUT[1]

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The factors determining cardiac output in man are incompletely understood. In this light, recent studies on vasodepressor syncope in this laboratory have suggested the presence in the upright subject of factors limiting the cardiac output response (1). In an attempt to investigate the nature of such factors, the present study on the effects of drug induced tachycardia in recumbent and upright subjects was undertaken. Atropine was selected as the experimental drug because of its well known cardio-accelerator effects, which in recumbent subjects is associated with little or no alteration in stroke volume. Additional observations have been made on modifications of the response to atropine during the peripheral pooling of blood with venous occlusive tourniquets and during anti-gravity suit inflation.

METHOD

The subjects were normal male university students, with ages from 18 to 25 years. The cardiac output was determined by the dye dilution method of Hamilton, Moore, Kinsman, and Spurling (2), as modified by Doyle, Wilson, Lépine, and Warren (3). In all studies a steady state, as determined by constancy of arterial pressure and pulse rate on monitored records, was achieved before the time of cardiac output determinations. Stroke volume was calculated from the pulse rate during the interval of the cardiac output determinations. Arterial and central venous pressures were obtained by strain gauge and recorded on a photographic multichannel recording system. The fourth intercostal space 5 cm. below the angle of the sternum was the reference point for all pressure determinations. Mean pressures were obtained electronically. A single balloon, half-body anti-gravity suit of the type reported by Beckman, Slaughter, and Wood (4) was employed in the studies of the effect of body compression. Inflation was maintained at 70 mm. Hg body pressure for five minutes before cardiac output determinations were performed in the control and postatropine states.

Mean circulation time and "central" blood volume was calculated by the Hamilton method from the dye dilution curves. Statistical analysis of the data was performed according to the methods described by Snedecor (5).

RESULTS

Effects of atropine in recumbent and passive erect postures

Two groups of six subjects each were studied, one in the recumbent and the other in the passive posture tilted head-up at 60 degrees. In all studies, control cardiac output determinations in the appropriate posture were made. Ten to fifteen minutes following the control determination, two milligrams of atropine sulfate were administered intravenously (catheter) over a one minute period, and cardiac output again determined one to three minutes after completion of the injection. In all subjects, tachycardia was in evidence at the time of this determination. In the studies performed with subjects tilted head-up at 60 degrees, the control and postatropine cardiac output determinations were performed during the same time intervals following assumption of the tilted position (usually three to six minutes). Arterial and central venous pressures were recorded continuously, except for interruptions during injection and collection periods.

The data are summarized in Tables I and II. In the recumbent position, striking increases in cardiac index and pulse rate were noted. The mean stroke volume was not altered significantly from control values. A fall in central venous pressure was noted in the four subjects in whom it was studied. Mean arterial pressure tended to rise slightly. The reductions in calculated total peripheral resistance can be attributed predominantly to the cardiac output changes.

In contrast to the changes noted with the subject in the recumbent position, the absolute and relative cardiac output elevations following atro-

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TABLE I

Effect of atropine on the cardiac index in recumbency and tilted head-up at 60 degrees

Recumbent				60° head-up tilt			
Subject		Cardiac index *L./min./M.²*	% change	Subject		Cardiac index *L./min./M.²*	% change
J. M.	B*	2.81		J. M.	B	2.55	
	A	5.19	+ 85		A	2.88	+13
J. G.	B	2.90		C. H.	B	2.32	
	A	4.53	+ 56		A	2.72	+17
D. M.	B	3.00		D. M.	B	2.86	
	A	6.71	+124		A	3.14	+10
A. Q.	B	2.94		R. K.	B	3.21	
	A	5.17	+ 76		A	3.21	0
P. B.	B	2.83		H. S.	B	2.01	
	A	5.31	+ 88		A	1.95	− 3
P. O.	B	2.91		J. C.	B	2.39	
	A	3.72	+ 28		A	2.96	+24
Average	B	2.90			B	2.56	
	A	5.06	+ 76		A	2.79	+10
p		<0.01				>0.05	

* B = Before atropine administration.
A = One to three minutes after atropine administration.

TABLE II

Hemodynamic effects of atropine administration in recumbency and tilted head-up at 60 degrees

Recumbent									
Subject		Cardiac output (*L./min.*)	Heart rate (*beats/min.*)	Stroke volume (*ml.*)	Mean arterial pressure (*mm. Hg*)	Total peripheral resistance (*units*)*	Central venous pressure (*cm. H_2O*)	Mean circulation time (*seconds*)	"Central" blood volume (*ml.*)
J. M.	B†	5.93	60	99	82	1,189	5	18.8	1,856
	A	10.96	120	91	90	656	−2	11.0	2,006
J. G.	B	6.20	64	98	76	980		22.6	2,337
	A	9.69	98	99	80	826		15.7	2,539
D. M.	B	5.70	68	84	87	1,220		25.7	2,440
	A	12.74	120	106	83	520		16.9	3,593
A. Q.	B	5.24	56	94	80	1,220	8	20.7	1,808
	A	9.20	94	98	93	808	4	14.1	2,162
P. B.	B	6.25	64	98	85	1,087	−1	20.8	2,169
	A	11.72	94	125	101	689	−4	15.5	3,024
P. O.	B	5.86	72	81	81	1,104	0	18.1	1,770
	A	7.48	120	62	89	951	−4	16.4	2,042
Average	B	5.86	64	92	79	1,133	3.0	21.1	2,063
	A	10.30	108	97	86	742	−1.5	14.9	2,561
S.D.‡		0.76	4.7	7.1	2.8	87		1.0	167
p		<0.01	<0.01	>0.5	<0.05	<0.01		<0.01	<0.05

* Dyne seconds times $cm.^{-5}$.
† B = Before atropine administration.
A = One to three minutes after atropine administration.
‡ Refers to standard error of the difference between means.

TABLE II—*Continued*

Subject		Cardiac output (*L./min.*)	Heart rate (*beats/min.*)	Stroke volume (*ml.*)	Mean arterial pressure (*mm. Hg*)	Total peripheral resistance (*units*)*	Central venous pressure (*cm. H_2O*)	Mean circulation time (*seconds*)	"Central" blood volume (*ml.*)
		60° head-up tilt							
J. M.	B	5.36	74	72	92	1,371	0	23.8	2,128
	A	5.84	136	43	95	1,300	0	23.1	2,248
C. H.	B	4.70	78	50	90	1,530		18.0	1,410
	A	5.52	148	37	85	1,231		16.8	1,546
D. M.	B	5.43	70	77	80	1,177		18.9	1,710
	A	5.96	138	43	80	1,073		16.5	1,639
R. K.	B	6.45	104	62	71	879		14.5	1,561
	A	6.45	168	36	69	854		20.2	2,174
H. S.	B	3.68	94	39	81	1,759	0	20.1	1,233
	A	3.58	152	24	85	1,846	−1	22.8	1,360
J. C.	B	4.37	60	73	87	1,591		21.2	1,543
	A	5.41	132	41	88	1,299		16.0	1,444
Average	B	5.00	80	64	84	1,385		19.4	1,598
	A	5.46	145	38	84	1,267		19.2	1,735
S.D.‡		0.18	2.2	4.2	1.7	62		1.6	104
p		>0.05	<0.01	<0.01	>0.9	>0.1		>0.9	>0.2

pine administration were significantly less in subjects in the passive erect posture ($p < 0.01$). The cardiac acceleration following atropine in this group was of greater relative and absolute magnitude, and the mean stroke volume fell significantly. Central venous pressure fell slightly in one of two subjects in whom it was studied. Mean arterial pressure and calculated peripheral resistance were not significantly altered.

"Central" blood volume was significantly higher in the recumbent subjects ($p < 0.05$). Following atropine, the mean central blood volume rose in the recumbent group, but did not change significantly in the tilted subjects.

Effects of anti-gravity suit inflation

Four studies were performed in which cardiac output was determined successively tilted head-up at 60 degrees, in the same position during sustained anti-gravity suit inflation (70 mm. Hg body pressure for five minutes), and tilted head-up at 60 degrees during sustained anti-gravity suit inflation one to three minutes following the intravenous administration of two milligrams of atropine sulfate. The data are summarized in Table III.

Sustained anti-gravity suit inflation resulted in slight increases in cardiac output in three of the four subjects with significant increase in stroke volume and "central" blood volume ($p < 0.05$). The increases in cardiac output following atropine in the vertical posture during sustained compression of the lower abdomen and lower extremities were significantly greater than the changes in cardiac output following atropine in the vertical posture alone ($p < 0.01$). The magnitude of these increases fell midway between those in the vertical and recumbent postures.

The pulse rate, calculated stroke volume, and "central" blood volumes following atropine administration in the above subjects likewise fell midway between the results in the vertical and recumbent subjects.

Effects of peripheral blood pooling

In order to study a component part of the postural reaction, the effect of peripheral pooling of blood was studied in five subjects. Blood pooling was produced by placing pneumatic cuffs high on three extremities and inflating to diastolic pressure levels. Cardiac output determinations were performed in the following sequence: 1) con-

TABLE III

Response to atropine during head-up tilt as modified by anti-gravity suit inflation

Subject		Cardiac index L./min./M.²	% change*	Heart rate beats/min.	% change*	Stroke volume ml./beat	% change*	"Central" blood volume ml.	% change*
J. K.	T†	2.85		80		67		1,491	
	T+G	3.81		84		85		2,111	
	T+G+A	5.21	+37	136	+62	72	−15	2,360	+12
P. R.	T	2.08		80		47		963	
	T+G	2.66		62		78		1,460	
	T+G+A	4.15	+56	108	+74	70	−10	1,495	+ 2
D. M.	T	2.59		88		55		1,387	
	T+G	3.01		79		72		1,640	
	T+G+A	4.40	+46	136	+72	61	−15	1,803	+10
P. B.	T	2.31		75		68		1,615	
	T+G	2.19		63		77		1,750	
	T+G+A	2.84	+30	104	+65	60	−22	1,969	+13
Average	T	2.46		81		59		1,362	
	T+G	2.92		72		78		1,740	
	T+G+A	4.15	+42	121	+68	66	−16	1,907	+ 9
p*		<0.01		<0.01		<0.01		<0.05	

* Refers to change from T plus G following atropine administration.
† T = Tilted head-up at 60 degrees.
G = During anti-gravity suit inflation.
A = One to three minutes after atropine administration.

TABLE IV

The effects of peripheral pooling of blood on the response to atropine in recumbent subjects

Subject		Cardiac index L./min./M.² Cuffs off	Cardiac index L./min./M.² Cuffs on	Heart rate beats/min. Cuffs off	Heart rate beats/min. Cuffs on	Stroke volume ml./beat Cuffs off	Stroke volume ml./beat Cuffs on	Central venous pressure cm. H_2O Cuffs off	Central venous pressure cm. H_2O Cuffs on	"Central" blood volume ml. Cuffs off	"Central" blood volume ml. Cuffs on
E. W.	B*	2.35	2.10	64	66	73	64	4.0	−3.0	1,659	1,607
	A	4.14	2.85	124	130	67	44	−2.5	−3.5	2,169	2,027
C. H.	B	2.34	2.41	50	54	94	89	2.0	0.0	1,890	1,683
	A	5.17	3.46	112	106	92	65	−4.0	−4.5	2,565	2,197
E. F.	B	2.63	2.15	48	48	105	85	3.0	1.0	2,048	2,078
	A	4.94	3.13	106	106	89	57	1.5	−1.5	2,622	1,947
D. M.	B	3.00	2.36	68	66	85	69	1.0	−2.0	2,066	1,663
	A	4.51	3.29	112	126	77	50	−3.0	−5.0	2,120	2,030
D. C.	B	3.87	3.46	62	70	119	94	3.0	−2.5	2,208	2,169
	A	5.65	3.41	120	136	89	47	−2.0	−3.0	2,500	2,026
Average	B	2.84	2.50	58	61	95	80	2.6	−1.3	1,974	1,840
	A	4.88	3.23	115	121	83	53	−2.0	−3.5	2,395	2,045
S.D.†		0.24	0.20	3.2	2.5	5.0	5.5	0.9	0.8	111	142
p		<0.01	<0.05	<0.01	<0.01	<0.05	<0.01	<0.01	<0.05	<0.01	>0.2

* B = Before atropine administration.
A = After atropine administration.
† S.D. refers to standard error of the difference between mean values.

trol observations before cuff inflation, 2) during the tenth to twelfth minute of cuff inflation, 3) two to three minutes following atropine sulfate administration during a comparable period of cuff inflation, and 4) six to eight minutes after atropine administration, but following deflation of the cuffs. The atropine was administered intravenously in two milligram doses as in the previous studies. In this manner the effects of venous pooling on the hemodynamic alterations induced by atropine were ascertained.

The data are summarized in Table IV. Sustained cuff inflation resulted in slight falls in cardiac index in four of the five subjects, and a slight decrease in stroke volume in all the subjects. Heart rate remained virtually unchanged while central venous pressure fell consistently. The "central" blood volume tended to be diminished during cuff inflation.

The effects of atropine when the pneumatic cuffs were not inflated were similar to the changes in the previous group of recumbent subjects. A comparison of the atropine effects before and during cuff inflation reveals a significant diminution in the cardiac output and stroke volume response during the peripheral pooling of blood ($p < 0.01$), associated with only slight differences in the degree of tachycardia. Central venous pressure fell consistently following atropine administration, the magnitude of these falls being less during cuff inflation. The increases in "central" blood volume following atropine were of lesser degree during cuff inflation.

DISCUSSION

Employing a variety of techniques, numerous investigators have reported that the cardiac output falls when man assumes the passive upright posture (6–8). On analysis in theory, these observed decreases in cardiac output might occur as the result of either reduced demands on the circulation, or limited ability of the heart to respond in the vertical stance. Oxygen consumption in the upright posture has been found to increase in most instances (7, 9), suggesting increased rather than decreased metabolic demands. If the diminished cardiac minute volume during standing reflects a limitation on the cardiac output in this position, a study of the response to an agent which ordinarily elevates cardiac output might serve to illuminate this factor more clearly. Accordingly, the effect of atropine has been studied. Although there have been somewhat conflicting experimental data reported, it appeared clear that the effect of rather large doses of atropine given intravenously would significantly elevate the cardiac output of the human subject in the recumbent position (10–12). The control observations of the present study confirm this finding.

The most striking feature of the observations reported here is that with the subject in the tilted position, atropine no longer elicits an increase in cardiac output. Furthermore, the failure of atropine to increase cardiac output in this position can be in part reversed by the application of anti-gravity suit compression. Similar reductions in the cardio-stimulatory effects of atropine are observed during the peripheral pooling of blood with venous occlusive tourniquets. Explanations for these phenomena must be considered.

When man assumes the upright posture, there is a considerable shift of blood to the lower part of the body, and a depletion of the central venous reservoir (13, 14). The decline in cardiac output, observed under these conditions, is associated with a decreased pressure in the right atrium (15), and evidence indicative of a decreased rate of ventricular filling (16). It is under these circumstances that tachycardia fails to produce an increased cardiac output. This observation is of particular interest in light of the suggestion that the central venous volume plays a strategic role as an immediate source of blood to sustain any sudden increase in cardiac output (14). In the recumbent posture, it would appear that more than adequate blood is available to meet the demands of a sudden tachycardia. Atrial pressure is relatively high and cardiac filling occurs rapidly. On assuming the upright position, the thoracic venous reservoir is depleted, ventricular filling is slower, and now stroke volume falls as the heart rate is suddenly increased. The capacity of the heart to increase its output becomes more directly dependent upon the systemic venous inflow. Under these circumstances, factors which increase venous inflow by diminishing gravitational effects, such as body compression by the anti-gravity-suit, restore cardiac responsiveness.

It is interesting to note that a higher pulse rate appears folowing atropine in the tilted individual,

as compared with the recumbent one. It would appear possible that the diminished stroke volume following atropine in the upright subjects could be related to the greater degree of tachycardia, the heart rate now being beyond an optimum level for ventricular filling. The observations on the effects of blood pooling, however, demonstrate a similar response to atropine but without significant differences in heart rate.

Consideration has been given to actions of atropine other than its effect on heart rate. It is possible, for instance, that atropine enhances peripheral blood pooling in the upright subject and in this manner further depletes the central reservoir. Such a peripheral action of atropine has not been demonstrated to our knowledge. The observations on the central blood volume and central venous pressure in the tilted subjects do not support such a formulation. However, if additional pooling did occur in the upright subject it would fortuitously complement the design of these experiments and would not alter their interpretation.

The atropine induced increases in cardiac output are primarily the result of rate alterations with practically no change in stroke volume. Other substances such as isoproterenol (Isuprel®) appear to produce increases in cardiac output by a more direct action on the myocardium. Studies in this laboratory on the effects of isoproterenol have demonstrated only slight diminution in the cardiac output response to this agent with subjects in the upright position (17). These observations would indicate that the limitations imposed on cardiac responsiveness by a diminished central reservoir may in part be superseded by alterations in ventricular dynamics.

Although there has been considerable debate in the literature on the validity of the determinations of central blood volume from dye injection data, the changes in calculated central volume ("Q" value) in the upright posture, during anti-gravity suit inflation and with peripheral blood pooling, were generally consistent with those expected from knowledge of the blood volume shifts induced in these situations (3). It is of particular interest that under the experimental situations when the cardiac output increased significantly, an elevated calculated central volume also appeared. The exact location between catheter tip and sampling site (including all temporally equidistant points) where this increase occurs unfortunately cannot be ascertained by the present methods. It is of further interest that the measured pressure at the point of dye injection (superior vena cava) consistently fell when the cardiac output and central volume increased. The exact meaning of these observations remains obscure at this time and their interpretation with respect to the central venous reservoir thesis outlined above can only be speculative. It should be emphasized, however, that the central reservoir of blood immediately available to the heart is probably but one compartment in the so-called central blood volume calculated from dye injection data, and that each may change independently during hemodynamic alterations. Studies are presently in progress in an attempt to delineate better the nature of these pressure and volume phenomena.

SUMMARY AND CONCLUSIONS

1. The cardiac output response to tachycardia induced by the intravenous administration of atropine sulfate has been studied in twelve normal subjects in recumbency and tilted head-up at 60 degrees. In four instances the effects of anti-gravity suit compression on the response to atropine in tilted subjects were studied. In five subjects the effects of peripheral venous pooling of blood on the response to atropine were also observed.

2. Significant increases in cardiac output, primarily the result of increased heart rate with little change in mean stroke volume, occurred in recumbent subjects. Only slight elevation of cardiac index, despite even greater tachycardia associated with a fall in mean stroke volume, occurred in the tilted group. Similar results following atropine were observed during peripheral pooling of blood. Sustained anti-gravity suit inflation restored, in part, the cardiac responsiveness to atropine in tilted subjects.

3. These data support the thesis that the central venous reservoir is an important determinant of cardiac responsiveness.

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THE PRESSURE-VOLUME RESPONSES OF HUMAN FOREARM VEINS DURING EPINEPHRINE AND NOREPINEPHRINE INFUSIONS [1, 2]

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There has been some speculation concerning the importance of active constriction of the veins in the production of changes in the distribution of the blood volume in man (2). The veins are elastic and they undoubtedly exert some influence on blood volume distribution by their passive resistance to the distending force of intraluminal pressure. Recent observations show that forearm veins do constrict actively (3–8) in response to various stimuli. There is little direct quantitative information, however, to demonstrate that such a mechanism is capable of shifting significant amounts of blood out of the extremities. This study was undertaken to determine whether or not the venous constriction induced by epinephrine or norepinephrine infusion is sufficient to move important quantities of blood from the limbs.

METHODS

The plethysmographic determination of forearm venous volume requires the measurement of forearm venous distensibility by the method of Litter, Wood, and Wilkins (7, 9). For making this measurement, the forearm segment is enclosed in a tall plethysmograph and water is added to a level such that the pressure on the arm is greater than venous pressure but less than diastolic arterial pressure. The arterial inflow continues and drives venous pressure to a height greater than that of the water column. The difference between the internal and external pressure is the *effective* venous pressure. Under these conditions this is a small positive value less than 1.0 mm. Hg—regarded for practical purposes as zero pressure (7). The venous volume at this effective pressure also is small and constant (10). After the limb is fixed, a pneumatic cuff about the arm proximal to the plethysmograph is inflated in 1.0 mm. Hg increments until the first perceptible increase in limb segment volume occurs. From this point of zero effective pressure, an additional pressure of 30 mm. Hg is applied to the arm in 5.0 mm. increments. This raises *effective* pressure in the forearm segment veins from 0 to 30 mm. Hg by the same increments of 5.0 mm. The increase in forearm venous volume caused by each pressure increment is recorded (Figure 1). A pressure-volume curve is constructed by plotting each volume level achieved at inflow-outflow equilibrium, expressed in ml. per 100 ml. of forearm tissue, against the corresponding level of effective venous pressure (Figure 2). The curve so obtained is convex toward the volume axis. If venous distensibility decreases (Figure 2), a subsequent curve will fall below the control curve, nearer the pressure axis. Conversely, if venous distensibility increases, the subsequent curve falls above the control curve, nearer the volume axis. The final point on the curve, the volume which exists at an effective pressure of 30 mm. Hg, is termed arbitrarily the venous distensibility.

The pressure-volume curve obtained in this manner expresses the volume to which the forearm venous system is distended by any level of effective venous pressure between 0 and 30 mm. Hg. Assuming that venous distensibility is the same in both arms, the natural venous volume of the forearm segment is the volume coordinate of that point on the curve which corresponds to the natural venous pressure (effective venous pressure) in the unencumbered forearm. This procedure for measurement of forearm venous volume was employed in a recent investigation by Wood and Eckstein (6).

PROCEDURES

Twenty-four experiments were performed on 18 healthy men 23 to 28 years old. The subjects were studied in the supine position with room temperature maintained at 80° F. They were nude except for shorts and a sheet covering the trunk and legs. The right forearm segment was enclosed in the plethysmograph. The arm was placed in the most comfortable position after it had been determined that varying arm position with respect to heart level did not alter venous distensibility. The plethysmographic water temperature was 89° F. An arterial occluding cuff was applied to the wrist for inflation before each volume measurement. The arm cuff was placed so that increments of pressure did not alter the

[1] Read in part by title at the Forty-ninth Annual Meeting of the American Society for Clinical Investigation in Atlantic City, N. J., May 6, 1957 (1).

[2] Supported by a research grant (H-2644) from the National Heart Institute of the Public Health Service, and aided by a grant from the Iowa Heart Association.

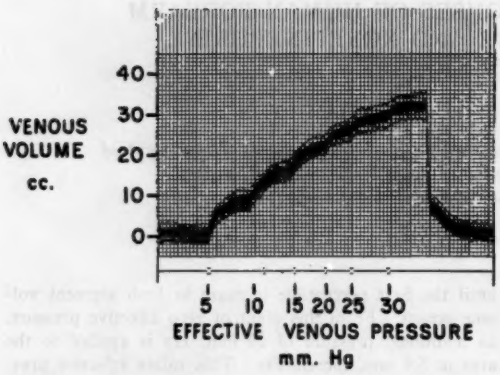

FIG. 1. CHANGES IN FOREARM VENOUS VOLUME PRODUCED BY KNOWN INCREMENTS OF EFFECTIVE VENOUS PRESSURE

position of the limb segment in relation to the plethysmograph. Forearm volume changes were measured with a Statham 0 to 5.0 cm. Hg pressure transducer which sensed changes in the height of the water in a vertical cylinder attached to the top of the plethysmograph. The diameter of the cylinder was such that maximal volume increases within the plethysmograph raised the water level 1.5 to 2.0 cm. The maximal error in effective pressure produced by this displacement was about 1.5 mm. Hg. This occurred only at the peak of some volume records. Since the change in volume for a 5.0 mm. Hg increment in effective pressure was small near the end of the volume record (Figure 1), this maximal pressure error caused only a negligible error in the measurement of limb volume above the baseline.

During the control period venous distensibility was measured intermittently in the right forearm. Simultaneous venous pressure measurements were made in the left antecubital vein with a Statham 0 to 5.0 cm. Hg pressure transducer. Volume and pressure were recorded with a Sanborn direct-writing oscillograph. Arterial blood pressure was determined by means of a sphygmomanometer between venous pressure measurements, with a cuff on the arm proximal to the needle. Pulse rate was determined by palpation of a peripheral artery. After values for venous distensibility, venous pressure, pulse rate and arterial pressure had become stable, epinephrine or norepinephrine was infused into a foot vein at a constant rate with a calibrated pump. Epinephrine chloride was delivered at either 7.5 or 15.0 μg. per minute. Norepinephrine bitartrate was delivered at 15.0 or 30.0 μg. per minute. The baseline venous volume of the forearm was recorded continuously during the initial portion of the infusion period. Occasional, very small reductions in baseline venous volume were noted, but none of these exceeded 0.1 ml. per 100 ml. of forearm tissue.[3] Five to seven minutes of infusion were required for venous pressure, pulse rate and arterial pressure to become stable at new levels. After stabilization, one or two measurements of venous distensibility were made. The infusion then was stopped and intermittent observations were made until control values returned. In certain experiments the epinephrine infusion was repeated at the same rate and for the same duration as in the initial infusion period. In other experiments epinephrine was infused a second time with the same rate and for the same duration after a single intravenous injection of 5.0 mg. of the adrenergic blocking agent phentolamine methanesulfonate (Regitine®).[4]

Following the experiments, venous pressure-volume curves were constructed. The natural venous volume existing during each measurement of distensibility was obtained by drawing a line perpendicular to the pressure axis from the natural venous pressure value (Figure 2). The volume coordinate of the point at which this line intersected the curve was taken to be the natural venous volume. Control values for venous distensibility, volume and pressure are averages of the final three control measurements. Infusion values are single measurements obtained during epinephrine or norepinephrine infusion. If more than one set of measurements were made during infusion, the first set is reported.

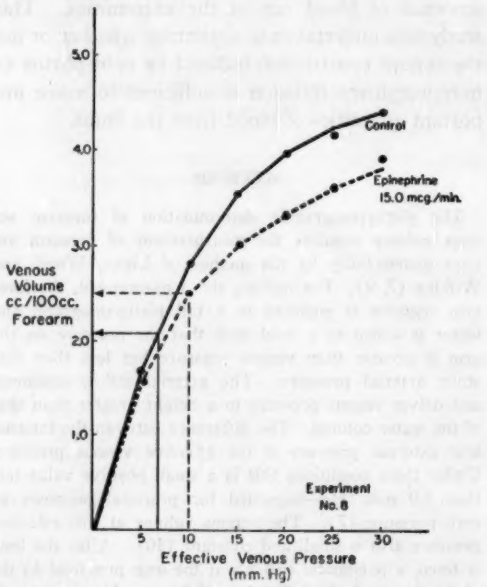

FIG. 2. VENOUS PRESSURE-VOLUME CURVES OBTAINED DURING A CONTROL PERIOD AND DURING AN EPINEPHRINE INFUSION WITH THE FOREARM AT ATRIAL LEVEL

[3] J. E. Wood and J. W. Eckstein in 1956 found occasional reductions in the baseline venous volume which ranged to 0.7 ml. per 100 ml. of forearm tissue when norepinephrine base was infused at 30.0 μg. per minute (11).

[4] Supplied by Ciba Pharmaceutical Products, Inc.

RESULTS

Epinephrine infusion with left forearm at atrial level

In the first group of eight experiments (Table I) epinephrine was infused first at 7.5 μg. per minute and repeated at 15.0 μg. per minute after control values had been reestablished. In each experiment venous distensibility decreased and venous pressure increased during the infusion periods. Venous distensibility averaged 3.9 ml. per 100 ml. of forearm tissue during control periods, and fell to an average of 3.2 during infusion at 7.5 μg. per minute. At an infusion rate of 15.0 μg. per minute the average value fell to 2.8 ml. per 100 ml. Venous pressure averaged 7.2 mm. Hg during control periods, rose to an average of 8.9 at an infusion rate of 7.5 μg. per minute and rose still further to an average of 10.0 at an infusion rate of 15.0 μg. per minute. In many instances the pressure-volume curves obtained during epinephrine infusion fell very close to the control curves for the first 10 to 15 mm. Hg increase in effective venous pressure (Figure 2). Beyond these levels the epinephrine curve moved away from the control curve toward the pressure axis. The natural pressure in the veins with the arm at atrial level (10.0 cm. above the back) often increased out of proportion to the decrease in venous distensibility so that at times blood appeared to shift into the forearm during epinephrine infusion. This relationship between pressure and distensibility with the arm at atrial level resulted in small, directionally inconsistent changes in natural forearm venous volume with infusions of 7.5 or 15.0 μg. per minute. There was essentially no change from the average control value of 1.6 ml. per 100 ml. of forearm tissue at either infusion rate.

Epinephrine infusion with left forearm dependent

In order to examine further and to quantitate more satisfactorily the volume shift of blood from the forearm veins which could be expected (on the basis of the preceding experiments) at higher natural venous pressure levels, the routine was changed in the second group of 11 experiments (Table II). Epinephrine was infused only at 15.0 μg. per minute and venous pressure was measured with the forearm approximately 14 cm. below

TABLE I

Forearm venous responses to epinephrine infusion—Forearm at atrial level

Experiment number	Control			Epinephrine 7.5 μg./min.			Epinephrine 15.0 μg./min.			After epinephrine		
	Venous pressure	Venous distensibility	Venous volume	Venous pressure	Venous distensibility	Venous volume	Venous pressure	Venous distensibility	Venous volume	Venous pressure	Venous distensibility	Venous volume
	mm. Hg	*ml./100 ml.*	*ml./100 ml.*	*mm. Hg*	*ml./100 ml.*	*ml./100 ml.*	*mm. Hg*	*ml./100 ml.*	*ml./100 ml.*	*mm. Hg*	*ml./100 ml.*	*ml./100 ml.*
1	7.7	4.4	2.1	8.1	4.1	1.9	9.6	2.9	1.9	8.0	4.8	2.0
2	5.9	4.5	1.8	9.4	3.6	2.2	11.5	2.8	1.5	5.9	4.2	1.6
3	8.0	3.9	1.6	8.9	2.3	1.0	9.3	1.8	0.9	7.4	3.7	1.2
4	8.5	3.8	1.6	10.4	3.3	1.6	10.4	3.0	1.6	7.0	3.5	1.4
5	7.3	3.0	1.4	9.6	2.3	1.3	10.0	2.2	1.0	5.9	2.7	1.2
6	4.9	4.2	0.9	5.2	3.6	0.7	6.0	3.3	1.0	4.5	4.3	1.0
7	8.6	3.0	1.3	10.0	2.5	1.6	13.5	2.3	1.6	8.5	3.6	1.4
8	6.5	4.4	2.1	9.3	4.1	2.4	10.0	3.9	2.5	6.3	4.6	2.5
Average	7.2	3.9	1.6	8.9	3.2	1.6	10.0	2.8	1.5	6.7	3.9	1.5
Standard deviation	1.31	0.61	0.39	1.63	0.76	0.58	2.11	0.67	0.54	1.30	0.68	0.49

TABLE II

Forearm venous responses to epinephrine infusion—Forearm dependent

Experiment number	Control			Epinephrine 15.0 μg./min.			After epinephrine		
	Venous pressure	Venous distensibility	Venous volume	Venous pressure	Venous distensibility	Venous volume	Venous pressure	Venous distensibility	Venous volume
	mm. Hg	*ml./100 ml.*	*ml./100 ml.*	*mm. Hg*	*ml./100 ml.*	*ml./100 ml.*	*mm. Hg*	*ml./100 ml.*	*ml./100 ml*
9	16.7	4.2	3.3	20.3	2.1	1.7	16.8	4.2	3.5
10	14.4	4.2	2.9	17.3	3.1	2.3	15.3	4.3	3.2
11	19.0	3.8	3.1	24.8	2.5	2.3	18.5	3.2	2.8
12	20.1	4.1	3.4	23.4	3.1	2.7	20.1	4.1	3.5
13	20.5	2.7	2.2	22.7	2.1	1.8	20.0	2.9	2.5
14	20.6	3.7	2.9	24.3	2.7	2.3	20.6	3.4	2.8
15	19.0	3.1	2.5	22.6	1.9	1.6	19.9	3.2	2.7
16	19.9	4.0	3.3	22.5	2.7	2.3	20.3	4.8	4.1
17	20.8	3.1	2.6	26.2	1.8	1.6	20.5	2.8	2.3
18	19.8	4.6	4.0	25.1	2.3	2.1	20.8	4.3	3.7
19	18.2	3.7	3.0	22.5	1.9	1.6	18.0	3.5	2.7
Average	19.0	3.7	3.0	23.0	2.4	2.0	19.2	3.8	3.1
Standard deviation	1.92	0.57	0.49	2.30	0.47	0.38	1.80	0.67	0.56

atrial level. Again venous distensibility decreased and venous pressure increased in each experiment during the infusion. Venous distensibility averaged 3.7 ml. per 100 ml. of forearm tissue during control periods and fell to an average of 2.4 during the infusion. Venous pressure averaged 19.0 mm. Hg during control periods and increased to an average of 23.0 during the infusion. In each of these experiments, in which natural venous pressure was in the physiologic range existing normally

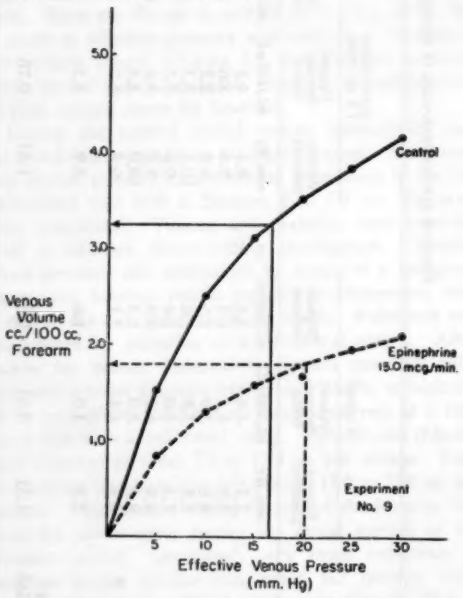

FIG. 3. VENOUS PRESSURE-VOLUME CURVES OBTAINED DURING A CONTROL PERIOD AND DURING AN EPINEPHRINE INFUSION WITH THE FOREARM DEPENDENT

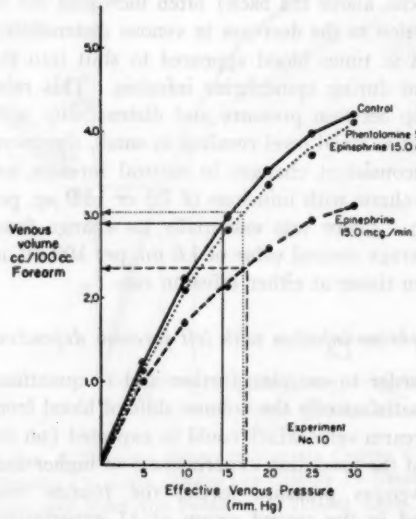

FIG. 4. VENOUS PRESSURE-VOLUME CURVES OBTAINED DURING A CONTROL PERIOD, DURING AN EPINEPHRINE INFUSION, AND DURING AN EPINEPHRINE INFUSION FOLLOWING PHENTOLAMINE WITH THE FOREARM DEPENDENT

in dependent forearms, the relationship between venous distensibility and pressure was such that epinephrine infusion resulted consistently in a shift of blood out of the forearm veins (Figures 3 and 4). The average venous volume during control periods was 3.0 ml. per 100 ml. of forearm tissue and fell to 2.0 during epinephrine infusion—an average volume shift of 33 per cent.

Norepinephrine infusion with left forearm dependent

In five experiments (Table III), norepinephrine bitartrate was infused at 15.0 μg. per minute and repeated at 30.0 μg. per minute after control values had been reestablished. Venous pressure was measured with the left forearm approximately 14 cm. below atrial level. Venous distensibility decreased and venous pressure increased in each case during the infusion. Venous distensibility averaged 3.9 ml. per 100 ml. of forearm tissue during control periods and fell to an average of 3.4 during infusion at 15.0 μg. per minute and to an average of 2.3 at an infusion rate of 30.0 μg. per minute. Venous pressure averaged 18.3 mm. Hg during control periods, rose to an average of 22.2 at an infusion rate of 15.0 μg. per minute, and rose still further to an average of 25.4 at an infusion rate of 30.0 μg. per minute. The venous volume averaged 3.1 ml. per 100 ml. of forearm tissue during control periods and fell to an average of 2.8 at an infusion rate of 15.0 μg. per minute. At an infusion rate of 30.0 μg. per minute the average venous volume fell to 2.0 ml. per 100 ml.—an average volume shift of 35 per cent.

Comparison between venous responses to epinephrine and norepinephrine infusions

The venous responses to epinephrine chloride infusion at 15.0 μg. per minute and the venous responses to norepinephrine bitartrate infusion at 30.0 μg. per minute may be compared since these infusion rates provide approximately equivalent amounts of sympatheticomimetic amine bases and since both groups of experiments were carried out with the forearm dependent (Tables II and III). Epinephrine infusion caused an average increase in venous pressure of 3.9 ± 1.19 mm. Hg, while norepinephrine infusion caused an average venous

TABLE III

Forearm venous responses to norepinephrine infusion—Forearm dependent

Experiment number	Control			Norepinephrine 15.0 μg./min.			Norepinephrine 30.0 μg./min.			After norepinephrine		
	Venous pressure	Venous distensibility	Venous volume	Venous pressure	Venous distensibility	Venous volume	Venous pressure	Venous distensibility	Venous volume	Venous pressure	Venous distensibility	Venous volume
	mm. Hg	*ml./100 ml.*	*ml./100 ml.*	*mm. Hg*	*ml./100 ml.*	*ml./100 ml.*	*mm. Hg*	*ml./100 ml.*	*ml./100 ml.*	*mm. Hg*	*ml./100 ml.*	*ml./100 ml.*
20	19.3	3.1	2.5	24.5	2.7	2.4	27.5	2.1	2.0	19.0	3.1	2.4
21	18.7	4.8	3.9	23.3	4.3	3.7	27.3	2.4	2.2	18.3	4.6	3.7
22	17.8	3.4	2.8	20.8	3.2	2.2	23.1	2.2	1.8	18.0	3.4	2.8
23	20.5	4.2	3.6	21.7	3.4	2.9	27.2	2.5	2.3	21.5	4.3	3.6
24	15.0	4.2	2.8	20.6	3.5	2.8	22.1	2.4	1.7	15.2	4.4	2.9
Average	18.3	3.9	3.1	22.2	3.4	2.8	25.4	2.3	2.0	18.4	4.0	3.1
Standard deviation	2.07	0.69	0.60	1.63	0.58	0.58	2.62	0.52	0.26	2.26	0.67	0.56

TABLE IV

Forearm venous responses to epinephrine infusion—Effects of repeated epinephrine infusion

Experiment number	Control			Epinephrine 15.0 μg./min.			Repeat epinephrine 15.0 μg./min.		
	Venous pressure	Venous distensibility	Venous volume	Venous pressure	Venous distensibility	Venous volume	Venous pressure	Venous distensibility	Venous volume
	mm. Hg	*ml./100 ml.*	*ml./100 ml.*	*mm. Hg*	*ml./100 ml.*	*ml./100 ml.*	*mm. Hg*	*ml./100 ml.*	*ml./100 ml.*
9	16.7	4.2	3.3	19.1	2.6	2.0	19.8	2.8	2.4
11	19.0	3.8	3.1	24.8	2.5	2.3	23.8	2.5	2.3
12	20.1	4.1	3.4	23.4	3.1	2.7	24.1	2.8	2.7
15	19.0	3.1	2.5	22.6	1.9	1.6	21.6	2.5	2.2
17	20.8	3.1	2.6	25.8	1.9	1.8	27.1	1.7	1.6
18	19.8	4.6	4.0	25.1	2.3	2.1	26.6	2.3	2.1
Average	19.3	3.8	3.2	23.4	2.4	2.1	23.8	2.4	2.2
Standard deviation	1.42	0.61	0.56	2.28	0.46	0.39	2.82	0.41	0.37

pressure increase of 7.2 ± 1.31. Epinephrine caused an average decrease in venous distensibility of 1.3 ± 0.53 ml. per 100 ml. of forearm tissue, while norepinephrine caused an average decrease of 1.6 ± 0.55. Epinephrine caused an average fall in venous volume of 1.0 ± 0.46 ml. per 100 ml., while norepinephrine caused an average fall of 1.1 ± 0.45. Despite the fact that norepinephrine caused a much greater rise in venous pressure than did epinephrine, the venous volume changes produced by these two materials were very similar since the slightly greater fall in venous distensibility with norepinephrine was sufficient to overcome the effect of the greater increase in distending pressure.

Epinephrine infusion repeated with and without prior administration of phentolamine

In six experiments, the epinephrine infusion was repeated at the same rate and for the same duration as the initial infusion. The venous pressure, distensibility and volume responses to epinephrine were found to be reproducible during repeated infusion (Table IV). In eight other experiments, the epinephrine infusion was repeated under the same conditions after a single intravenous injection of 5.0 mg. of phentolamine. In each case the decrease in venous distensibility and the increase in venous pressure were much less when epinephrine was infused after phentolamine administration (Table V). In the three phentola-

TABLE V

Forearm venous responses to epinephrine infusion—Effects of repeated epinephrine infusion after phentolamine

Experiment number	Control			Epinephrine 15.0 μg./min.			Repeat epinephrine 15.0 μg./min. (after phentolamine)		
	Venous pressure	Venous distensibility	Venous volume	Venous pressure	Venous distensibility	Venous volume	Venous pressure	Venous distensibility	Venous volume
	mm. Hg	*ml./100 ml.*	*ml./100 ml.*	*mm. Hg*	*ml./100 ml.*	*ml./100 ml.*	*mm. Hg*	*ml./100 ml.*	*ml./100 ml.*
2	5.9	4.5	1.8	11.5	2.8	1.5	8.1	3.6	1.4
3	8.0	3.9	1.6	9.3	1.8	0.9	6.7	3.4	1.0
4	8.5	3.8	1.6	10.4	3.0	1.6	7.8	3.5	1.9
5	7.3	3.0	1.4	10.0	2.2	1.0	5.9	2.5	1.2
6	4.9	4.2	0.9	6.0	3.3	1.0	4.3	4.3	0.9
10	14.4	4.2	2.9	17.3	3.1	2.3	16.8	4.0	3.1
13	20.5	2.7	2.2	22.7	2.1	1.8	21.0	2.4	2.2
14	20.6	3.7	2.9	24.3	2.7	2.3	20.8	3.0	2.4
Average	11.5	3.8	1.9	13.9	2.6	1.6	11.4	3.3	1.8
Standard deviation	6.40	0.72	0.71	6.70	0.53	0.57	6.93	0.67	0.80

mine experiments (Nos. 10, 13 and 14) in which venous pressure was measured with the forearm dependent, the phentolamine likewise appeared to prevent the fall in forearm venous volume which had been produced regularly by epinephrine infusion alone (Figure 4).

The reduction in the venous responses to epinephrine infusion following phentolamine administration are probably due to the adrenergic blocking action of the latter drug rather than to its direct vasodilating action (12), since in two experiments phentolamine administration (5.0 mg.) alone caused no change in venous distensibility.

DISCUSSION

The peripheral venous system may be considered an extension of the central vascular reservoir (great veins and pulmonary vessels) from which blood is pumped by the heart. The volume and pressure within the central reservoir are important determinants of the cardiac output, and under steady conditions they are in equilibrium with it. An increase in cardiac output must be associated with a pressure-volume readjustment within the reservoir which increases the availability of blood to the heart. Such a readjustment also must require a reduction in capacity of the peripheral venous system and a shift of blood centrally, *i.e.*, an increased *venous return*. Of the many factors involved in the regulation of venous return, probably the least is known about the quantitative importance of active venous constriction. Since the veins probably contain at least 75 per cent of the blood volume (2), even a relatively slight amount of venous contraction might be expected to move appreciable amounts of blood.

If one assumes that the pressure-volume responses of the veins of the arms and legs are similar, it becomes possible to estimate the total amount of blood shifted out of the extremities by the venous constriction caused by epinephrine and norepinephrine infusion. Litter and Wood (10) found the average vascular volume of the leg of a supine subject (effective venous pressure about 8 mm. Hg) to be approximately 3.5 ml. per 100 ml. of leg tissue. If the average volume of each upper extremity is four liters (10) and each lower extremity 13 liters (13), the average total blood volume of the four limbs would be approximately 1,200 ml. This value is in agreement with that of Ebert and Stead (14), who found the average blood volume of one upper and two lower extremities to be 900 ml. Litter and Wood (9) also found that at an effective venous pressure of 30 mm. Hg the average venous volume of the leg was 4.75 ml. per 100 ml. We found that at an effective venous pressure of 30 mm. Hg, the average venous volume of the forearm was 3.7 ml. per 100 ml. (Table I). Sjöstrand (15) pointed out that an average of more than 600 ml. of blood was shifted from the lower extremities when standing subjects assumed the reclining position. Furthermore, Ebert and Stead (14) found that an average of 720 ml. of blood could be pooled in one upper and two lower extremities by venous tourniquets. From these figures it is apparent that the venous volume of four *dependent* extremities probably exceeds 1,500 ml. Under the conditions of our experiments, epinephrine and norepinephrine infusions caused shifts of blood out of the forearm which averaged 34 per cent. Based on the observations cited above, this shift in terms of the four extremities would amount to more than 500 ml. This quantity of blood, returned from the limbs alone, would represent an appreciable fraction of the estimated normal central blood volume, which is probably less than 1,200 ml. (16).

Our contention that much of this blood shifts centrally is supported by the work of Ranges and Bradley (17), who found increases in right atrial pressure in man following administration of epinephrine; and by the observations of Bondurant, Hickam, and Isley (18), who found increases in central venous pressure in man during norepinephrine infusion. Furthermore, Rashkind, Lewis, Henderson, Heiman, and Dietrick (19) found an increase in the volume of a central venous reservoir (which collected the total venous return) in dogs in response to the administration of epinephrine and norepinephrine. Freis and Rose (20) also demonstrated an increase in central reservoir volume and an associated increase in right ventricular output during the administration of norepinephrine to dogs. Peripheral venous constriction, with the resulting increase in venous return, may contribute to the increase in cardiac output observed in normal man during epinephrine infusion (21) and in dogs in early experimental hemor-

rhagic shock during treatment with norepinephrine (22).

Wood, Litter, and Wilkins (7) demonstrated that reductions in forearm venous distensibility are present in patients with congestive heart failure who have elevated venous pressure. This reduction in distensibility, compared with values observed in compensated patients, averaged 1.5 ml. per 100 ml. This diminution in venous distensibility in a chronic condition corresponds closely with our reduction values of 1.3 and 1.6 ml. per 100 ml. for epinephrine and norepinephrine effects, respectively, during acute administration. In the experimental situation with these two hormones, the shift of blood out of the extremities, presumably centrad, probably serves to augment the cardiac output. From a teleologic standpoint, it appears that the same venous response would be generally desirable in congestive heart failure, inasmuch as the primary physiologic abnormality is a subnormal cardiac output. However, in the severe pulmonary vascular engorgement, associated with acute left ventricular decompensation, it might aggravate the abnormality. Venisection or application of tourniquets to the extremities probably relieves the emergency situation by diminishing the effectiveness of this natural venomotor response in the presence of a normal right ventricular myocardium.

SUMMARY

Forearm venous distensibility, pressure and volume were measured during 24 experimental sessions in man before and during epinephrine infusion, during epinephrine infusion following phentolamine administration, following phentolamine administration alone, and before and during norepinephrine infusion. The following observations were made:

1. Forearm venous distensibility decreased while venous pressure increased during epinephrine infusion in each of 19 experiments. Epinephrine infusions repeated under the same conditions produced quantitatively similar venous pressure and distensibility responses in each of six experiments.

2. These epinephrine responses were smaller in each of eight experiments when phentolamine was administered prior to repetition of the epinephrine infusion. In two other experiments there was no change in venous distensibility when phentolamine was administered alone.

3. Norepinephrine caused a much greater rise in venous pressure and a slightly greater fall in venous distensibility than did epinephrine in comparable doses.

4. With subjects at rest in the supine position with the forearm at atrial levels, epinephrine produced only small inconsistent changes in forearm venous volume.

5. When the forearm was dependent, both epinephrine and norepinephrine infusions regularly caused large shifts of blood of comparable magnitude out of the forearm veins. It is suggested that such venous volume shifts from the dependent extremities in man's upright position to the central venous reservoir may be important in augmenting the cardiac output at times of physiologic need.

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UNBOUND AMINO ACID CONCENTRATIONS IN HUMAN BLOOD PLASMAS

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Studies of small molecules in plasma have indicated the importance of defining concentrations in terms of both "unbound" and "total." For example, McLean and Hastings (1) showed calcium was both bound and free in plasma, but that the free form was regulatory in the body. Further, Davis (2) found it was the unbound concentrations of the sulfonamides in the plasma which were active. In addition to these, cholesterol, fatty acids, bilirubin, estriol, chloride and many others have been observed to bind with various affinities to the plasma proteins [see review by Surgenor (3)].

The amino acids in blood may be considered to exist in one or more of the following forms:

a) combined into proteins or other substances by covalent linkages (peptide bonds and possibly other stable bonds).
b) combined with proteins or other substances by weak forces.
c) present within the various formed elements of the blood (erythrocytes, leucocytes, platelets).
d) present in plasma as individual unbound molecules.

The unbound plasma amino acid concentrations have been considered as the concentrations present in form d), and have been measured experimentally after equilibration at nearly physiological condition of pH of freshly prepared plasma across cellophane membranes known to be freely permeable to the amino acid molecules. Many of the earlier estimates have failed to remove completely the formed elements of the blood to exclude form c), or have involved long durations of manipulation and/or temperature changes so that proteolytic and other enzymes may have produced changes in the concentration of unbound amino acids.

The total free plasma amino acid concentrations may be considered to be the concentrations present in forms b) and d), and have been measured experimentally after precipitation of freshly prepared plasma by agents such as trichloroacetic acid, picric acid, and phosphotungstic acid. These substances cause large changes in pH, and denaturation of some or all of the proteins present.

In this report, a method is described for measuring the amino acid levels in the unbound form d). Employment of nonwettable surfaces for blood collection, immediate cooling of the specimens, early cold centrifugation, rapid dialysis, and avoidance of changes in pH or other destructive steps in the analysis of the dialysates are believed to be necessary for this purpose.

Concentrations of the amino acids are determined by paper chromatography. In order to secure satisfactory chromatograms, the developing solvents are saturated with buffered sodium chloride and the papers are pretreated with salt. Under these conditions, the physiological salts of the plasma do not interfere with chromatography. A desalting step with its possible destructive effect is avoided. Salt-saturated developing solutions have been reported previously (4), but the use of such solvents with salt-treated papers does not appear to have been published.

METHODS

Preparation of protein free concentrates

A syringe and needle treated to make their surfaces nonwettable (5) and containing one-third ml. of heparin solution (1,000 U.S.P. units per ml.) are used to collect 25 ml. of blood. The blood is placed immediately in a vinyl plastic bag which is submerged in ice water for

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prompt transportation to the laboratory. The plasma is separated by centrifugation in a refrigerated centrifuge at 1,750 times gravity for 40 minutes and removed from the bag by compression. Ten ml. is dialyzed at 2° C. for two hours against 65 ml. of distilled water, using the thin layer technique of Seegers (6). A sealed glass tube, which is weighted with lead pellets and placed inside the dialysis bag, is 16 mm. in outside diameter and 215 mm. in length. The dialysis membrane is Nojax Casing, size 23/32, obtained from the Visking Corporation, Chicago, Illinois. This arrangement produces a layer of plasma 1 to 2 mm. thick. Agitation is accomplished by suspending the membrane from a stirring motor assembly controlled to alternate between 30 second periods of rotation at 90 r.p.m. and equal periods of nonrotation. This method of agitation was suggested by Dr. N. A. Saroff, Naval Research Institute, Bethesda, Maryland. Following the dialysis, the dialysate is stirred for 10 minutes with 1 ml. of wet, mixed anion exchange resin, Rohm and Haas XE-114, mesh size 50-100, of which one part is the ethylenediaminetetraacetate salt and three parts are the chloride salt. This step reduces distortions of some of the amino acid zones on the chromatograms, presumably through removal of bivalent cations. It has been found not to remove any of the amino acids.

Sixty ml. of the dialysate is then concentrated to dryness from the frozen state. At this point the residue may be safely stored at −10° C. For applications to the papers the residue is dissolved in 0.4 ml. of water. The solution volume with this amount of water is found to be approximatey 0.43 ml. for dialysates derived from normal plasma.

Pretreatment of papers

Whatman No. 1 and Whatman No. 4 papers, size 18¼ by 22½ inches, are used. The pretreatment consists of dipping the papers in a borate buffer solution, pH 8, containing 3.5 Gm. H_3BO_3, 0.25 Gm. NaOH, and 30 Gm. NaCl per liter of water and suspending the papers in a nonwrinkled condition to dry. Manipulation of the wet papers is facilitated by attaching one end to a wooden clamp consisting of two slats (1 by 1 by 24 inches) held together by two bolts and winged nuts.

Application of dialysate concentrates and standard solutions to the papers

One dimensional chromatograms are run in the long direction of the paper. A separate paper is prepared for each staining reagent except Sakaguchi's. This reagent is applied to the chromatogram developed for and stained with Ehrlich's reagent after the results of the latter test have been recorded. The pretreated papers are placed in a support to facilitate applications of the amino acid solutions (7). These are applied to the papers with a 3 μl. pipette in an arrangement similar to that used for quantitative analysis by McFarren and Mills (8), except that aliquots of 3, 6 and 12 μl. of the unknown, and aliquots of 3, 6, 9, and 12 μl. of the standard solution are used. The composition of the standard solution is listed in Column A of Table I.

TABLE I

Composition and chromatographic characteristics of the amino acid mixture

	Standard solution mM/L. (A)	R_F values	
		Solvent A (B)	Solvent B (C)
Alanine	4.3	0.10	0.20
Allantoin		0.14	
α-amino n-butyric	0.5	0.22	0.50
Arginine	1.0	0.06	0.44
Asparatate		0.007	0.02
Citrulline		0.055	0.08
Cystine		0.006(0.20)*	0.05
Glutamate	1.7	0.01	0.04
Glutamine	6.8	0.04	0.22
Glycine	3.4	0.053	0.07
Histidine	1.7	0.065	0.54
Isoleucine	1.0	0.46	0.75
Leucine	1.5	0.50	0.75
Lysine	2.2	0.032	0.20
Methionine	0.5	0.29	0.68
Ornithine	1.4	0.028	0.12
Phenylalanine	0.8	0.40	0.82
Proline	2.4	0.14	0.71
Serine	1.7	0.053	0.05
Taurine		0.06	0.04
Threonine	2.2	0.08	0.14
Tryptophan	0.7	0.35	0.77
Tyrosine	1.2	0.26	0.49
Urea	68.0	0.38	
Valine	3.0	0.32	0.60

* This amino acid frequently splits into two zones in this solvent.

Development

After the applications have been made, the papers are hydrated by placing them in a cold room until they have adsorbed enough moisture to make them feel damp. Following hydration, the papers are immediately placed in chromatographic chambers prepared for each solvent, as indicated below, and descending development started. The preferred solvent for determining each amino acid is listed under the heading Staining Techniques. The solvents used are prepared as follows:

Solvent A. Isopropanol, reagent grade, is mixed with borate buffer solution of the same composition as used for pretreatment of the papers in the ratio of volumes 80:20. Solid sodium chloride is then added with agitation until a small amount of salt remains undissolved in the bottom of the flask. The organic layer is decanted, part is poured over adsorbent papers attached to the sides of the chamber to hasten saturation of the vapor phase, and the remainder is used for development of chromatograms. It has been found advantageous to start development immediately after the solvent has been poured over the papers on the sides of the chambers.

Solvent B. Liquified phenol (Mallinckrodt, Gilt Label) is mixed with borate buffer solution and concentrated ammonium hydroxide in the volume ratios of 90.5:9:0.5.

The solvent is saturated with sodium chloride and the organic layer is decanted for use in development. With this solvent, improved chromatograms are obtained by saturating the vapor phase in the chamber with water rather than with the developing solvent. This is accomplished by covering the bottom of the chamber with water.

The migration rates (R_F values) of amino acids and several other substances in these solvents are listed in Columns B and C of Table I. Solvent A is the more stable of the two and gives the more reproducible results. It is used in preference to solvent B when choice permits.

Staining techniques

After development, the papers are dried in a mechanically vented oven at 60° C. prior to staining.

Ninhydrin-collidine reagent. This stain is prepared by dissolving 0.5 Gm. of ninhydrin in 100 ml. of a mixture of acetone, glacial acetic acid, lactic acid (85 per cent), methylcellosolve, water and collidine (2,4,6-trimethylpyridine) in the volume proportions 66 : 9.5 : 0.5 : 10 : 10 : 4, respectively. The collidine is added immediately before use. The papers are dipped in this reagent and heated at 60° C. for 15 minutes for color development. The lactic acid enhances the ninhydrin stain, and the collidine causes color contrast in the stains of the amino acids (9). The following amino acids or combination of amino acids are determined by this reagent:

Alanine, arginine, glutamic acid, glutamine, lysine, ornithine, threonine and a combined value for *serine* and *glycine* are determined on a Whatman No. 4 paper developed for 64 hours in solvent A.

Glycine is determined on a Whatman No. 1 paper developed for eight hours in solvent B.

α-amino n-butyric acid, isoleucine, leucine and a combined value for *methionine, tryptophan* and *valine* are determined on a Whatman No. 1 paper developed for 16 hours in solvent A. A corrected value for valine is obtained by subtracting the tryptophan and methionine values found with the special reagents described below.

Special reagents. The low plasma concentration of some amino acids and the low staining intensities of others with the ninhydrin-collidine reagent makes it desirable to use more sensitive staining reagents for their determination. These tests are run on Whatman No. 1 papers developed for 16 hours in solvent A.

Histidine and *tyrosine* are determined by Pauly's reagent as modified by Bolling, Sober, and Block (10).

Tryptophan and *urea* are determined by Ehrlich's reagent as modified by Smith (11). Allantoin is also stained by this reagent and its color appears occasionally in some analyses.

Arginine may be determined by Sakaguchi's reagent as modified by Acher and Crocker (12). This is sometimes helpful because arginine is in low concentration in plasma and cannot always be determined by the ninhydrin-collidine reagent. When this occurs, Sakaguchi's reagent is applied over the stain of Ehrlich's reagent, making it unnecessary to prepare an additional paper.

Proline, phenylalanine and *tyrosine* are determined by isatin, using a modification of the method of Saifer and Oreskes (13). Five-tenths Gm. isatin is dissolved in a mixture of acetone, acetic acid and water in the volume proportions of 88 : 4 : 8, respectively. The paper is dipped in this reagent and heated for one hour at 60° C. for color development. Colors from other amino acids do not interfere under these conditions. The tyrosine values determined by this reagent are usually averaged with those obtained by Pauly's reagent.

Methionine is determined by the platinum reagent of Toennies and Kolb (14). Cystine is also observed when this reagent is used, but elongation of its zone with occasional splitting into two zones makes its determination inexact.

Quantitation

The amounts present on the chromatograms are determined visually by comparison of the size and intensities of the zones of the unknowns with those of the standards. The values observed are multiplied by 1.04 to compensate for losses occurring during analysis. The principal loss is the amount absorbed on the dialyzing membrane.

An alternate method of quantitation is to measure the maximum intensities of the zones on the paper photometrically, as described by Block, Durrum, and Zweig (15). This method in general is not recommended for the present types of chromatograms. Whereas its accuracy is comparable to that obtained visually for amino acid zones of high intensity, it is less accurate for weakly pigmented or irregular zones. Further, the variation in color of zones brought out by the ninhydrin-collidine and special reagents complicates photometry by requiring measurements at a diversity of wavelengths. Such color differences assist visual evaluation by clearer delineation of the zones. Photometry is also slower. For example, chromatograms from 14 dialysates require approximately 40 hours for photometric analysis, whereas visual analysis can be accomplished in 6 to 8 hours.

RESULTS AND DISCUSSION

Amino acid binding to non-dialyzable molecules in plasma

Amino acid binding was measured by recovery studies. A fresh sample of plasma was divided into 14 parts. Three parts were analyzed to establish the amino acid concentrations in the original plasma and 11 parts were analyzed after various mixtures of amino acids had been added. Recoveries were calculated and the data shown in Table II. With the exception of tryptophan and glutamic acid, no significant difference is indicated between the calculated values and the amounts found ($p > 0.06$). It should be noted

TABLE II

Recovery of amino acids added to plasma

	Average amounts added	Total amounts calculated present	Total amounts found	Standard deviation	Coefficient of variation	Per cent recovery
	μM/L.	μM/L.	μM/L.			
Alanine	133	352	350	18.0	5.2	99
α-amino n-butyric acid	16	30	29	2.4	8.5	96
Arginine	32	105	106	10.0	9.4	105
Glutamic acid	72	72	89	5.6	6.3	131
Glutamine	215	870	880	54.3	6.2	105
Glycine	111	293	303	22.4	7.4	109
Histidine	53	142	153	9.1	5.9	120
Isoleucine	32	87	85	7.8	9.2	92
Leucine	48	144	134	7.7	5.8	81
Lysine	70	228	240	19.3	8.1	117
Methionine	16	34	32	5.8	18.0	87
Ornithine	65	140	135	14.8	11.0	92
Phenylalanine	27	68	70	8.4	12.0	106
Proline	75	167	162	14.0	8.7	92
Threonine	70	170	166	17.9	10.8	94
Tryptophan	28	28	7		18.0*	<28
Tyrosine	37	87	81	8.0	10.0	83
Valine	96	248	243	9.7	4.0	95
Serine and glycine	161	507	510	35.7	7.0	101
Urea	2,136	6,320	6,440	354.0	5.5	105

* Calculated from other data.

that the amount of amino acids added averaged only 40 per cent of the total present. Recoveries calculated for these added amounts reflect experimental variation two and one half times larger than if recoveries had been calculated on the total present.

Tryptophan showed distinct binding. Approximately 70 per cent of the added tryptophan was absent from the dialysate, but could be recovered from the solution inside the bag if the proteins were precipitated with trichloroacetic acid. This observation explains the low tryptophan values

TABLE III

Comparison of amino acid concentrations in plasma obtained by two techniques

	Values by rapid dialysis and salt-saturated chromatography solvents			Values from Stein and Moore (16)*	
	No.	Mean	Range	Mean	Range
		μM/L.	μM/L.	μM/L.	μM/L.
Alanine	15	320	200–450	383	338–418
α-amino n-butyric acid	15	20	10– 30	29	21– 34
Arginine	15	68	50– 96	87	70–111
Glutamic acid	15	<20	0– 25	48	29– 78
Glutamine	15	450	320–780		
Glycine	13	235	140–290	205	178–231
Histidine	15	88	60–110	74	51– 95
Isoleucine	14	57	35– 90	68	52–100
Leucine	15	79	50–105	129	108–175
Lysine	10	143	105–190	186	171–207
Methionine	13	28	10– 45	25	22– 29
Ornithine	5	58	46– 75	55	47– 61
Phenylalanine	14	56	35– 75	51	42– 58
Proline	15	142	100–180	205	160–292
Serine and glycine	15	320	270–390	312	274–340
Taurine	2	10	0– 20	44	33– 66
Threonine	15	135	100–180	117	102–144
Tryptophan	11	21	14– 26		
Tyrosine	15	70	35– 90	57	48– 80
Valine	15	174	110–220	246	202–317

* Converted from mg. per 100 ml.

obtained in the plasma analyses of the next section.

Glutamic acid was found only in those specimens to which it had been added. However, as shown by the table, the amount found was in excess of the amount added. This excess corresponds to a concentration of 17 μM in the original plasma, a value which is reasonable because concentrations less than 20 μM generally cannot be determined by the procedure used.

These recovery studies indicate that except for tryptophan the amino acids are not reversibly bound to the large molecules of plasma. It is possible that binding does occur between amino acids and small dialyzable molecules. However, chromatography of dialysates at neutral pH in a much concentrated form showed no characteristic variation of the R_F value of any amino acid and suggests that binding with smaller molecules probably does not occur to any appreciable extent.

Equilibrium binding studies will not indicate degradations of labile complexes when these occur irreversibly. Since such degradations are more likely to occur under acid conditions and least likely to occur at neutrality, analyses by both methods are compared in the next section.

Unbound amino acid levels in normal fasting plasmas

Fifteen normal, fasting plasmas were analyzed by methods described above and the results reported in Table III. For comparison, Stein and Moore's results of plasma from five fasting normal subjects, analyzed by picric acid deproteinization and a resin column effluent technique, are included (16). In addition to the amino acids listed in the table, Stein and Moore reported determinations of aspartic acid, asparagine, the methyl histidines, serine and cystine plus cysteine but did not report values for tryptophan and glutamine which were destroyed in their analysis. Neither cystine nor cysteine is determined separately. The other three substances, as well as citrulline, are not observed on the chromatograms, presumably because their concentrations are too low.

Values obtained by these two methods for many of the amino acids agree rather closely. Levels of alanine, glycine, histidine, isoleucine, methionine, phenylalanine, threonine, tyrosine and a combined value for serine and glycine are essentially the same. Somewhat less good agreement is shown between the values for α-amino n-butyric acid, arginine, leucine, lysine, proline and valine. The values of Stein and Moore are 25 to 60 per cent higher for these amino acids. In view of the variation in normal individuals and the small number of analyses reported, differences in these values are not of established significance.

The average glutamic acid concentration as determined by rapid dialysis and paper chromatography is, on the other hand, significantly less than the value reported by Stein and Moore, or, for that matter, significantly less than any other known report of glutamic acid concentrations in plasma. In the study of the 15 normal samples, a mean value less than 20 μM was indicated. Glutamic acid was found in only two of the analyses at levels of 20 and 25 μM. The average value reported by Stein and Moore of five analyses was 48 μM. Krebs, Eggleston, and Hems (17) reported an average value of 238 μM (3.5 mg. per 100 ml.). Walshe (18), using Dent's procedure of electrolytic desalting with paper chromatography, found glutamic acid in such high concentration it was considered a major component of the "free" amino acids in plasma. To explain these large differences, it seems likely that the technique of the latter investigators degraded a labile plasma substance, presumably by the acid conditions employed. Stein and Moore suggest that some glutamic acid may come from hydrolysis of glutamine in their analysis. It is also possible that glutamic acid may leak from the erythrocytes during the handling of whole blood.

Determinations of glutamine in this study, in which an average concentration of 450 μM was obtained, agree reasonably well with the values reported by Archibald (19), and Krebs, Eggleston, and Hems (17). These observers made determinations by use of glutaminase. They reported average values of 8.3 and 5.8 mg. per 100 ml. of plasma or 570 and 400 μM per liter, respectively.

Large differences occur between tryptophan values found in this study and values reported by Hier and Bergeim (20), and Johnson and Bergeim (21). They used microbiological assays on acid-deproteinized supernatant solutions. The mean unbound value for the 11 normal samples reported in Table III is 21 μM, whereas they obtained values in normal plasma of 55 μM (1.11 mg. per 100

TABLE IV

Effect of surgery on the plasma levels of unbound amino acids *

	Patients in good nutritional status			Patients emaciated			Mean percentage change in levels immediately post-operative observed in 16 patients by Everson and Fritschel (23)
	No. subjects	Mean levels before operation $\mu M/L.$ plasma	Mean percentage change in levels after operation	No. subjects	Mean levels before operation $\mu M/L.$ plasma	Mean percentage change in levels after operation	
Alanine	5	230	+ 2 ± 8†	3	120	+53 ± 4†	
α-amino n-butyric acid	3	30	−27 ± 13	2	25	−20 ± 16	
Arginine	5	80	−43 ± 8	3	47	+13 ± 9	−28
Glutamine	5	368	−32 ± 10	3	233	− 4 ± 6	
Histidine	5	94	−28 ± 11	3	100	−17 ± 21	−12
Leucine‡	5	170	−65 ± 6	3	113	−14 ± 11	− 8
Isoleucine‡	5			3			−23
Lysine	5	114	−33 ± 7	3	67	− 6 ± 10	−15
Methionine	3	40	−32 ± 5	3	20	− 5 ± 0	−26
Phenylalanine	2	65	−35 ± 16	1	50	0	−26
Proline	3	217	−42 ± 9	2	85	0 ± 12	
Serine and glycine	3	237	−27 ± 5	2	150	−10 ± 10	
Threonine	5	100	−22 ± 14	2	57	+17 ± 13	−30
Tryptophan	5	44	−37 ± 6	3	23	+ 9 ± 33	−40
Tyrosine	5	66	−41 ± 10	3	50	−14 ± 23	
Valine	5	254	−48 ± 3	3	127	+ 5 ± 3	−12
Average % change			−34			0	−20

* At the time this study was undertaken analyses for some of the amino acid had not been developed.
† Standard deviations of the mean percentage changes.
‡ Leucine and isoleucine values were combined in this study.

ml.) and 64 μM (1.3 mg. per 100 ml.), respectively. These differences in concentration arising from different procedures of analyses coincide with the observation made in the binding study of this amino acid in the previous section. It should be further noted that the levels of unbound tryptophan reported in Table III are probably high because the dilution occurring during dialysis tends to remove more tryptophan from the binding site. This binding is being further studied.

Another observation made in studying the unbound amino acid levels in normal plasma is the low levels of taurine. This substance has been reported present in plasma by other investigators (16, 22). In an effort to determine trace amounts of taurine, zones were eluted from several chromatograms, concentrated, and rechromatogramed in another solvent. By this technique, plasma taurine concentration was estimated at approximately 10 μM. In plasma from whole blood exposed to wettable surfaces, or plasma which had not been centrifuged adequately to remove most of the leucocytes and platelets, higher levels were obtained. These observations indicate that taurine may be derived from the leucocytes and platelets. In unpublished experiments of the authors, taurine has been found in very high levels in these cells.

Additional studies

The effect of surgery on the plasma levels of amino acids was studied in eight patients undergoing operations of one to three hours duration. Either cyclopropane, ether, or spinal anesthesia was administered. The patients' diagnoses were subacute cholecystitis, chronic cholelithiasis (two), varicose veins, umbilical hernia, carcinoma of the duodenum, burns complicated by chronic alcoholism, and severe burns, respectively. The first five patients were in reasonably good nutritional status. The latter three were emaciated, with the two burn patients undoubtedly in negative nitrogen balance. The patient with carcinoma of the duodenum had lost 60 pounds in the period six months preceeding the operation. Blood specimens were drawn immediately before and immediately after surgery.

The data from this study are summarized in Table IV. The patients are grouped according to their nutritional condition. The preoperative amino acid levels of those in good nutritional status are in the general range of the normals reported in Table III. In this group an average decrease in amino acid level of 34 per cent occurred after surgery. This decrease is in agree-

ment with the observations of Everson and Fritschel (23) in their study of the postoperative changes of the essential amino acids in 16 patients, although the magnitude of the change we observed is larger than theirs. With the emaciated group, a decrease was not observed. In this group the preoperative amino acid levels were low, and the additional stress of surgery did not further depress them.

Alanine showed anomalous behavior. Its postoperative concentration in relation to the other amino acids increased about 50 per cent in both groups of patients studied. This increase does not appear to have been reported previously.

SUMMARY

1. A technique is reported for assaying unbound amino acids in human plasma. Containers with nonwettable surfaces are used during collection and separation of the cells to avoid release of cell substance. The amino acids are separated from the plasma proteins by rapid dialysis and analyzed by paper chromatography in salt-saturated solvents on salt-saturated papers. This method avoids a desalting step. Physiological pH is maintained throughout and low temperatures maintained up to the start of the chromatograms. In addition to amino acids, urea may also be determined by this method.

2. Unbound concentrations of eighteen individual amino acids and a combined value for serine and glycine have been determined in plasma samples from fifteen normal subjects. With the exception of tryptophan and glutamic acid, agreement with other assays has been obtained.

3. The unbound concentration of glutamic acid and tryptophan are found to be very low in plasma. At physiological pH, tryptophan is bound to a nondialyzable plasma substance, probably protein, but is released upon precipitation of the proteins with trichloroacetic acid.

4. Recovery studies of amino acids added to plasma show that none were bound except tryptophan.

5. Plasma levels of amino acids of patients in good nutritional status dropped an average of 34 per cent immediately after surgery. In three emaciated patients whose preoperative levels were approximately 65 per cent of normal levels, no amino acid drop occurred after surgery.

6. Alanine showed anomalous behavior. In the normal patients, the alanine level remained the same while the average of all the amino acids decreased 34 per cent. In the emaciated patients, the alanine level increased 50 per cent while the average of all amino acids remained the same. Thus, alanine in relation to the other amino acids increased postoperatively in both groups of patients studied.

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THE MECHANICAL BEHAVIOR OF THE LUNGS IN HEALTHY ELDERLY PERSONS[1,2]

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In a previous study it was found that the elastic properties of lungs appeared to be unaffected by age in healthy adults 17 to 39 years old (1). The slope of the static volume-pressure curve of lungs in the resting tidal range of breathing was used as a measure of pulmonary compliance. The present study extends observations to adults 50 years and older. To obtain a more complete description of the elastic behavior of lungs, static transpulmonary pressure has been related to the total volume of gas in the lungs, and the range of volume change has been extended from that of tidal volume to inspiratory capacity. In addition, measurement has also been made of pulmonary compliance at various rates of breathing, and of pulmonary flow resistance. Finally, these data have been compared with findings in young adults.

MATERIAL AND METHODS

Eight male and twenty female volunteers, ranging in age from 50 to 89 years, were studied. All were apparently healthy and lived at home. Most of the women did their own housework, while four of the eight men were still employed in office work. They denied any recent or chronic cardiopulmonary disease, and had no untoward shortness of breath. Photofluorograms of their chests were considered to be "normal." Part of the data on young adults was obtained from previous studies (1, 2). Additional measurements were made on a group of young adult volunteers.

All measurements were made with the subjects seated. Intraesophageal pressure was used as an index of intrapleural pressure. It was obtained from a thin latex balloon, approximately 15 cm. in length, affixed to polyethylene tubing (3). The balloon was passed to the lower portion of the esophagus. An inductance manometer was used to measure differences in pressure between the esophagus and the mouthpiece (transpulmonary pressure). Pulmonary compliance was measured statically and dynamically, that is, during interrupted and spontaneous breathing. Air flow was interrupted by means of a solenoid valve located in the mouthpiece assembly; the pressure tap on the mouthpiece lay between the mouth and valve. Volume was recorded with a nine-liter capacity Benedict-Roth spirometer. A commutator on the spirometer wheel energized the solenoid valve at steps of 250 ml. (ATPS). The duration of valve closure was controlled by a time-delay circuit (1); closure lasted approximately three seconds. All static measurements of compliance were made during inspiration starting from the resting end-expiratory level.

For dynamic measurements of pulmonary compliance, volume changes were measured with a seven-liter capacity Krogh spirometer to which was attached a rotational transducer. The spirometer was connected to a bag-box system (4), enabling the subject to breathe air for periods lasting up to several minutes. Instantaneous flow rate was obtained by electrical differentiation of the volume signal. The three electrical signals (pressure, volume, flow rate) were amplified and transcribed by means of a direct-writing oscillograph.

During spontaneous breathing, pulmonary compliance was measured as the ratio of the change in volume (tidal volume) to the change in transpulmonary pressure between instants of zero air flow at the volume extremes of inspiration and expiration. Because variations occurred due to changes in intraesophageal pressure accompanying cardiac contraction, average values for compliance (and flow-resistance) during spontaneous breathing were calculated from at least 20 to 30 measurements.

Pulmonary flow-resistance was calculated in two ways. The first method is shown in Figure 1. Points were chosen in both the inspiratory and expiratory phases of a respiratory cycle when lung volumes were identical and flow rates were about maximal. In the tidal range of volume change the elastic component of transpulmonary pressure is the same at these instants. Accordingly, the observed change in pressure between these points relates solely to flow-resistance. The ratio of this pressure change to the corresponding change in flow between these points has been calculated. This value represents an "average" flow-resistance for inspiration and expiration.

In the second method, flow-resistance was measured separately for inspiration and expiration. To do this, values for instantaneous flow-resistive pressures were calculated as the difference between observed changes in transpulmonary pressure occurring between the start of inspiration (or expiration) and some point near maximal flow rate, and the corresponding elastic component

[1] This study was aided by a grant from The National Foundation for Infantile Paralysis, Inc.

[2] Presented in part before The National Tuberculosis Association, May 21 to 23, 1956, New York, and The American Physiological Society, September 4 to 7, 1956, Rochester, New York.

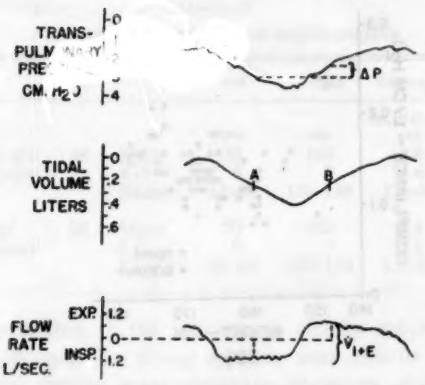

FIG. 1. AT IDENTICAL LUNG VOLUMES, A AND B, ΔP RELATES ONLY TO PULMONARY FLOW-RESISTANCE

"Average" pulmonary flow-resistance for a respiratory cycle may be expressed as:

$$R = \frac{\Delta P}{\dot{V}_I + \dot{V}_E} \text{ in } \frac{\text{cm. } H_2O}{\text{L./sec.}}$$

of transpulmonary pressure. The elastic component, in turn, was estimated from measurements of compliance and the volume change that had occurred between the two points.

Each measurement of flow-resistance has two components—airway, including upper airway and glottis in addition to tracheobronchial tree, and tissue viscous resistance. No attempt was made to measure these components separately. Finally, the assumption is implicit that in the range of air flow rates encountered during quiet breathing, flow-resistance is nearly constant.

The resting end-expiratory gas volume of the lungs (FRC) was measured by use of a helium closed-circuit technique (5). Subdivisions of the vital capacity were recorded on a spirometer, and the total lung capacity was calculated from the sum of the functional residual capacity and inspiratory capacity. All volumes were expressed at body temperature and pressure saturated (BTPS).

TABLE I

Pertinent physical characteristics of 28 healthy elderly subjects

	No.		Age	Height	Weight	B. S. A.*
			yrs.	*cm.*	*Kg.*	*M²*
Male	8	Mean	76	173	17.0	1.79
		S.D.	8	4	8.7	0.13
		Range	66–89	167–178	61.8–86.3	1.56–1.97
Female	20	Mean	67	163	61.6	1.66
		S.D.	8	5	7.8	0.17
		Range	50–81	150–170	39.1–74.5	1.34–1.83

* Body surface area calculated from Du Bois height-weight formula (19).

TABLE II

Mean pulmonary compliance, end-expiratory and maximal static inspiratory pressures, and lung volumes in healthy subjects

	No.	Age	Height	Compl.	End-exp. transpulm. pressure	Max. static insp. transpulm. pressure	Corresponding vol. change	I. C.*	E. R. V.†	Vital capacity		F. R. C.‡	R. V.§	T. L. C.‖
										Observed	Predicted			
		yrs.	*cm.*	*L./cm. H₂O*	*cm. H₂O*	*cm. H₂O*	*L.*	*L.*	*L.*	*L.*	%	*L.*	*L.*	*L.*
Younger subjects	11	Mean	166	0.150	4.2	30.4	2.30	2.51	1.35	3.86	125	2.93	1.58	5.44
		S.D.	7	0.027	1.6	7.4	0.36	0.45	0.26	0.59	18	0.54	0.42	0.79
		Range	158–178	0.08–0.18	2.5–6.5	21.5–45.0	1.70–2.80	1.95–3.49	0.93–1.75	2.88–5.24		2.45–3.81	1.18–2.39	4.38–7.39
Older subjects	21	Mean	166	0.131	4.8	20.7	1.78	2.06	1.06	3.13	116	3.67	2.60	5.73
		S.D.	7	0.038	2.0	6.4	0.46	0.61	0.46	0.71	16	0.80	0.50	1.11
		Range	150–178	0.08–0.23	1.5–8.5	12.5–37.0	1.01–2.84	0.83–3.43	0.18–2.06	1.90–4.47		2.50–5.23	1.80–3.73	3.96–7.58
		p		>0.1	>0.1	<0.01	<0.01	<0.05	<0.1	<0.01		<0.02	<0.01	<0.1

* Inspiratory capacity.
† Expiratory reserve volume.
‡ Functional residual capacity.
§ Residual volume.
‖ Total lung capacity.

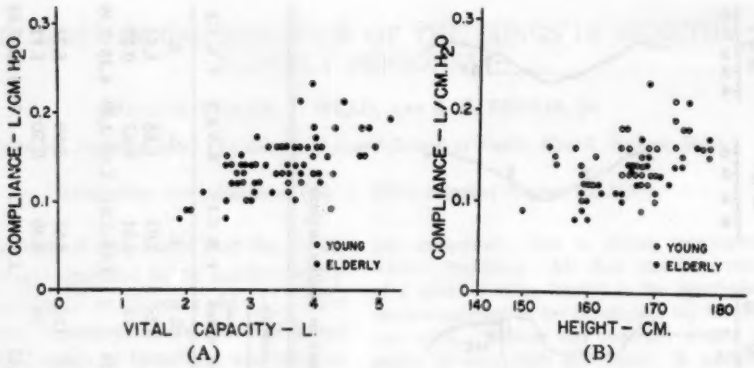

FIG. 2. COMPLIANCE IS PLOTTED AGAINST (A) VITAL CAPACITY AND (B) HEIGHT FOR 26 ELDERLY AND 41 YOUNG SUBJECTS

RESULTS

Average values for the pertinent physical characteristics of 28 elderly[3] subjects are shown in Table I.

Static measurements

In 26 of the elderly subjects, measurements of compliance were made during interrupted breathing starting from the end-expiratory relaxation volume, and compared with similar measurements in a group of 41 younger subjects of the same average height (1).[4] This measurement expresses the slope of the static volume-pressure characteristics of lungs in the range of normal tidal volume for the sitting position. Average values for compliance were 0.130 ± 0.036 L. per cm. H_2O among older subjects and 0.144 ± 0.036 L. per cm. H_2O among younger subjects; the difference was not significant. The correlation between compliance and either height or vital capacity was approximately the same in both age groups (Figures 2A and 2B).

Measurements of total lung capacity and subdivisions were made in 21 of the older and 11 of the younger subjects, each group being of the same average height. Average values are shown in Table II, along with values for compliance, static end-expiratory transpulmonary pressure, and transpulmonary pressure measured at the peak of maximal inspiration. The latter pressure was measured after gas flow had stopped and while the glottis was open. These data are plotted in Figure 3.

It will be noted that, whereas functional residual capacity was larger among elderly subjects ($p < 0.01$), static end-expiratory transpulmonary pressures were not significantly different for the two groups. These findings indicate that the average over-all static volume-pressure curve of the lungs

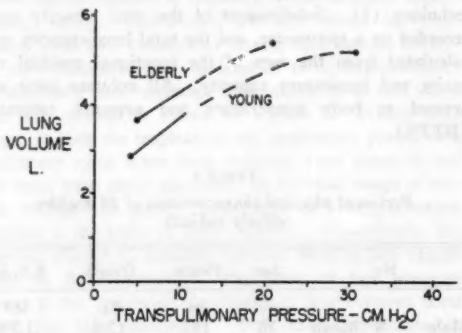

FIG. 3. AVERAGE VALUES FOR STATIC VOLUME-PRESSURE CHARACTERISTICS OF LUNGS ARE SHOWN FOR 21 OLDER AND 11 YOUNGER SUBJECTS

Points indicate measured volumes and pressures. Slopes of the solid lines indicate values for compliance computed from direct measurements. The broken lines are estimated values.

[3] "Elderly" is applied in this study to persons 50 years and older.

[4] Previously, no consistent difference was found in compliance between males and females when subjects of the same size were compared (1). For this reason the data on compliance, including those shown in Figures 2A and 2B, are not subdivided according to sex.

TABLE III

Pulmonary flow resistance in healthy subjects

	No.		Age	Height	Resistance
			yrs.	*cm.*	*cm. H_2O/L./sec.*
Younger subjects	28	Mean	28	168	1.9
		S.D.	6	9	0.6
		Range	18–47	154–189	1.2–3.4
Older subjects	28	Mean	70	165	2.8
		S.D.	9	7	0.8
		Range	50–89	150–178	1.3–4.4

was shifted to the left among elderly subjects. In addition, the young subjects were able to develop greater transpulmonary pressures during maximal inspiratory effort ($p < 0.01$). Despite this difference, total lung capacity was not significantly different for the two groups.

Flow-resistance measurements

In 28 young adults[5] the range of values for "combined" inspiratory and expiratory flow-resistance (see under Methods) was 1.2 to 3.4 cm. H_2O per L. per second, averaging 1.9 ± 0.6 cm. H_2O per L. per second (Table III); only one subject had a value for flow-resistance above 3.0 cm. H_2O per L. per second. Among the 28 elderly subjects, it varied between 1.3 and 4.4 cm. H_2O per L. per second, averaging 2.8 ± 0.8 cm. H_2O per L. per second; flow-resistance was higher than 3.0 cm. H_2O per L. per second in 10 of this group. The average value for the eight elderly males was

TABLE IV

Inspiratory and expiratory pulmonary flow resistance in healthy elderly subjects

	Inspiratory resistance	Expiratory resistance	Inspiratory resistance / Expiratory resistance
	cm. H_2O/L./sec.	*cm. H_2O/L./sec.*	
(a) Eighteen subjects having "combined" flow resistance* under 3.0 cm. H_2O/L./sec.			
Mean	2.0	3.4	0.6
S.D.	0.7	1.2	
Range	0.9–3.0	1.4–5.9	
(b) Ten subjects having "combined" flow resistance over 3.0 cm. H_2O/L./sec.			
Mean	3.1	4.9	0.6
S.D.	0.9	1.14	
Range	1.6–4.1	3.5–6.8	

* Defined in Methods.

[5] Ten of these subjects were reported before (2).

TABLE V

Mean pulmonary compliance, end-expiratory and maximal static inspiratory transpulmonary pressures and lung volumes in healthy elderly subjects having pulmonary flow-resistance below or above 3.0 cm. H_2O/L./sec.

Flow resistance	No.	Age	Height	Compl.	End-exp. transpulm. pressure	Max. static insp. transpulm. pressure	I. C.	E. R. V.	V. C.	F. R. C.	R. V.	T. L. C.
		yrs.	*cm.*	*L./cm. H_2O*	*cm. H_2O*	*cm. H_2O*	*L.*	*L.*	*L.*	*L.*	*L.*	*L.*
Below 3.0 cm. H_2O/L./sec.	14	72	166	0.137	5.0	19.9	1.73	1.50	3.23	3.80	2.62	5.85
		S.D.	11	7	0.042	2.2	5.9	0.44	0.49	0.70	0.75	0.49	1.07
		Range	50–89	150–178	0.09–0.23	1.5–10.0	14.0–37.0	1.01–2.35	0.74–2.46	2.09–4.47	2.51–5.23	1.97–3.73	4.69–7.58
Above 3.0 cm. H_2O/L./sec.	6	Mean	69	164	0.113	5.3	22.8	1.73	1.08	2.82	3.33	2.45	5.25
		S.D.	9	6	0.035	1.6	8.2	0.41	0.45	0.81	1.03	0.64	1.32
		Range	53–79	158–172	0.08–0.17	3.5–8.0	12.5–36.0	1.24–2.34	0.53–1.74	1.90–4.08	2.50–4.82	1.80–3.41	3.96–7.49

TABLE VI

Pulmonary compliance during interrupted, quiet and rapid breathing in healthy elderly subjects

Subject no.	Flow resistance	Interrupted breathing compl.	Quiet breathing		Rapid breathing	
			Rate	Compl.	Rate	Compl.
	cm. H_2O/L./sec.	*L./cm. H_2O*	*min.*	*L./cm. H_2O*	*min.*	*L./cm. H_2O*
5	2.1	0.17	14	0.17	74	0.16
13	2.2	0.11	13	0.14	33	0.12
25	2.2	0.12	16	0.13	52	0.09
26	2.7	0.13	10	0.12	48	0.08
	Mean	0.132		0.140		0.112
2	3.8	0.12	15	0.09	42	0.08
9	4.4	0.08	16	0.07	41	0.06
15	3.2	0.14	14	0.12	59	0.11
18	3.1	0.13	20	0.12	42	0.11
27	4.2	0.14	20	0.12	52	0.10
	Mean	0.122		0.104		0.092

2.5 ± 0.8 cm. H_2O per L. per second, and for the 20 elderly females, 2.9 ± 0.8 cm. H_2O per L. per second. To determine whether the higher values (over 3.0 cm. H_2O per L. per second) for flow-resistance were due to changes predominantly in one or the other phase of the respiratory cycle, flow-resistances were calculated separately for inspiration and expiration at identical lung volumes (Table IV). Inspiratory resistance measured about two thirds as much as expiratory resistance for the group whose "combined" flow-resistance was less than 3.0 cm. H_2O per L. per second, as well as for those with higher values. The ratio of inspiratory to expiratory flow-resistance was also calculated in six of the young subjects and found to be nearly 0.9. The latter value is roughly equal to that derived from the data of Fry, Ebert, Stead, and Brown (6) in healthy young adults at a flow rate of 1.0 L. per second, although higher than the value of 0.7 found by Attinger, Monroe, and Segal (7) at slow rates of breathing in the sitting position. It is felt that the number of observations in the present study is too small to draw a conclusion regarding the significance of the difference in ratios between the two age groups.

When the elderly subjects were divided into groups having flow-resistance below or above 3.0 cm. H_2O per L. per second, no significant differences were found in mean static compliance, subdivisions of total lung capacity (including F.R.C.), or static transpulmonary pressures, both at the end of normal expiration and following maximal inspiration (Table V).

Dynamic compliance measurements

Measurements of compliance were made in nine of the elderly subjects during quiet and rapid breathing, care being taken to prevent changes in end-expiratory lung volume and tidal volumes. The group was divided into those having flow-resistance either below 3.0 cm. H_2O per L. per second or above that value (Table VI). The group having lower flow-resistance showed no consistent change in compliance as the rate of breathing increased. The group having higher flow-resistance showed a progressive decline in compliance with increasing rates of breathing in all instances; the magnitude of change was small, however, when compared with that observed among patients having emphysema (2, 8–10).

Smoking habits of the elderly subjects

Twenty-one elderly subjects had not smoked; five had been regular smokers more than 10 years, three of cigarettes and two of cigars; two of the subjects were not questioned. Pulmonary flow-resistance was above 3.0 cm. H_2O per L. per second in only 6 of the 21 nonsmokers, whereas it was above this value in four of the five smokers. (One subject who smoked cigars had a value of 2.2 cm. H_2O per L. per second.) Despite this considerably greater incidence of elevated flow-resistance among smokers, it is felt that the group is too small to warrant a conclusion about the possible relation of smoking to changes in flow-resistance. With regard to other measurements, there was no apparent pattern of change in static

compliance or subdivisions of lung volume among the elderly smokers.

DISCUSSION

It should be emphasized that this study does not permit a distinction between changes inherent in the aging process and those which may represent the effects of repeated disease or injury to the lungs. According to Bates and Christie, "emphysema is a gross exaggeration of what happens to the lungs with advancing years" (11). They found a reduction in vital capacity and an increase in functional residual capacity in both healthy elderly persons and patients having emphysema. The changes accompanying aging were attributed to "fixation of the thoracic cage" and to reduced elastic recoil of the lungs, though no direct measurement of elastic properties of either structure was reported. The judgment of these authors gains support from findings in the present study. In general, the static volume-pressure curve of the lungs in elderly subjects was shifted to the left so that at any volume their lungs appeared to have diminished elastic recoil compared with those of young adults. This shift of the entire volume-pressure curve could be detected only by relating measurements of compliance to absolute gas volumes in the lungs and transpulmonary pressure. The fact that there was no significant difference in mean compliance in the range of tidal breathing between the two age groups emphasizes that this measurement expresses only a part of the over-all elastic behavior of the lungs.

In addition, there is evidence of an alteration in the elastic properties of the thorax which may be described as follows: The volume of gas remaining in the lungs at the end of expiration during quiet breathing is determined by the opposing elastic recoil of the lungs and thorax. An increase in this volume can result from either reduced elastic recoil of the lungs, increased elastic recoil of the thorax, or a combination of both. The relative contribution of the two forces should be reflected in the end-expiratory transpulmonary pressure. For example, if all of an increase in functional residual capacity were due to reduced elastic recoil of the lungs, end-expiratory transpulmonary pressure would decrease as the thorax approached its own "relaxation" volume. On the other hand, if all of an increase in functional residual capacity were attributable to increased tendency of the thorax to recoil "outward," end-expiratory transpulmonary pressure would increase. It follows that an increase in functional residual capacity which is unaccompanied by significant change in transpulmonary pressure is evidence for a combination of reduced elastic recoil of the lungs and increased thoracic elastic recoil. The observation that young and elderly subjects have substantially the same end-expiratory transpulmonary pressures during quiet breathing indicates that the larger functional residual capacity of older subjects results from a combination of diminished elastic recoil of the lungs and increased elastic recoil of the thorax. For the purpose of this discussion it has been assumed that the functional residual capacity is a "relaxation" volume not influenced by muscular contraction.

The thesis of Bates and Christie may be extended to include other attributes of the mechanical behavior of lungs. For example, it has been well established that pulmonary flow-resistance is increased in emphysema (2, 6, 8–10, 12), the change being greater during expiration; to a much smaller degree pulmonary flow-resistance, chiefly during expiration, was also elevated in some elderly subjects. In emphysema, dynamic pulmonary compliance falls rapidly as the respiratory rate increases (2, 8–10); among the elderly subjects, when flow-resistance was elevated, compliance did fall slightly but significantly as the rate of breathing rose, whereas changes in compliance were not consistent when flow-resistance was within the range encountered in young adults. A possible mechanism for dependence of compliance upon respiratory rate has been described, based on the mechanical implications of nonuniformity of flow-resistance and/or elastic changes within the lungs (2, 8). The authors concluded that alterations in the distribution of ventilation must occur in association with the reduction in dynamic compliance at increased respiratory rates. This conclusion is consistent with the observation of Greifenstein, King, Latch, and Comroe (13) who found with the use of single breath and pulmonary emptying rate tests that many healthy older subjects show evidence of a greater nonuniformity of ventilation than do young subjects.

It is apparent that the changes in mechanical behavior of lungs of elderly adults compared with young adults are in every instance qualitatively similar to those observed in emphysema. On the other hand, it should be noted that the magnitude of such changes that accompany aging is generally small, and in individual instances not detectable. It would seem that additional factors must play a role in the reduced performance of elderly subjects in such tests of maximal effort as the vital capacity (8, 13–18), and maximal breathing capacity (13, 16, 18). An attempt was made in the present study to estimate respiratory muscle strength by measuring the maximal pressure each subject could develop across an obstructed mouthpiece. The attempt was discontinued after a few studies because it was felt that most of the subjects did not understand the procedure well enough to cooperate satisfactorily. But apart from the question of muscular strength, it would appear that changes in elastic properties of the thorax might well account for much of the reduction in vital capacity. Evidence has already been presented suggesting that the thorax has an increased tendency to recoil outward, and that this together with diminished elastic recoil of the lungs accounts for the increased functional residual capacity of elderly subjects. The change in elastic behavior of the thorax would also be expected to result in an increase in residual volume of the lungs and a decrease in vital capacity (largely at the expense of the expiratory reserve) due to the fact that it was more difficult to compress the thorax. Table II shows that such changes in subdivisions of the total lung capacity were typical in the present study; they have been reported by other investigators as well (12–16).

SUMMARY

Physical properties of the lungs of 28 healthy subjects 50 years and older were measured in the sitting position. Values were related to measurements of total lung capacity and its subdivisions. When compared with a group of 11 healthy adults ranging in age from 22 to 47, the older subjects showed a shift in static volume-pressure curve of the lungs to the left. Pulmonary flow-resistance was above 3.0 cm. H_2O per L. per second in 10 out of 25 elderly subjects, and in only 1 out of 28 younger subjects. Among elderly subjects, dynamic compliance decreased consistently as the respiratory rate increased in those having values for pulmonary flow-resistance above 3.0 cm. H_2O per L. per second. These changes in physical properties of the lungs were similar in kind to, though much less than those found in, emphysema. They were associated with an increase in functional residual capacity, decrease in vital capacity, and no change in total lung capacity.

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CAPILLARY OBSERVATIONS IN PATIENTS WITH HEMORRHAGIC FEVER AND OTHER INFECTIOUS ILLNESSES [1]

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Although the capillary vascular system is known to be affected during many infectious illnesses (1–5), the *in vivo* study of the small blood vessels in man during infection has been almost entirely neglected. Certain infectious diseases are especially characterized by clinical syndromes which can be largely or entirely attributable to a generalized dysfunction of the vascular system (6–8); a study of the reaction of the small blood vessels in these states might therefore be particularly significant. One such illness, hemorrhagic fever, was selected initially for the *in vivo* study of the capillary alterations produced by infectious agents in humans. Hemorrhagic fever was first encountered by Western physicians during the Korean Campaign. A classical case usually proceeded through four rather distinct phases; namely, 1) an initial acutely febrile onset, persisting for an average of three to five days, 2) a hypotensive or shock phase often developing during defervescence and persisting from a few hours to several days, 3) a hypertensive oliguric stage lasting three to five days on the average, and 4) a diuretic phase merging into convalescence (9). The deeply flushed appearance, the petechiae and ecchymosis of the skin, conjunctiva and hard palate, and the strongly positive Rumpel-Leede test, all occurring early in the disease (10, 11), suggested the occurrence of a profound and generalized alteration in the function of the small blood vessels. The characteristic postmortem findings, consisting chiefly of intense generalized capillary dilatation, focal hemorrhages, and edema in the loose areolar tissues, strongly support this concept (12–14). Indeed many, if not most, of the clinical features which develop after the febrile phase can be accounted for as the consequences of generalized capillary dysfunction.

This report describes the changes detected during serial observations of the nail-fold capillary bed of such patients with hemorrhagic fever. The capillary alterations observed in patients with other infectious diseases who were studied in a similar manner are also described. The findings indicate that significant alterations of the capillary vessels may be observed directly during hemorrhagic fever and during certain other infectious illnesses, and that these capillary alterations can be correlated with the clinical course and pathological findings.

MATERIALS AND METHODS

Selection of patients

Random and serial capillary studies were performed on 190 consecutive United Nations soldiers admitted to the Forty-eighth Mobile Army Surgical Hospital in Korea during the fall of 1953 because of febrile disease. The subjects, ranging in age from 18 to 45, were usually transferred from Division Clearing Station during the third or fourth day of illness with the tentative diagnosis of "hemorrhagic fever, suspect." Eighty of this group were rejected because the observer was unable to visualize the nail-fold capillary vessels adequately (negroid pigmentation, thickened cuticles) or because of local pathologic alterations (clubbing, chronic inflammation, traumatization). The diagnosis of hemorrhagic fever was eventually confirmed in 71 patients; 39 patients were found to have various other acute febrile illnesses. Nine of a group of young male adult patients in the United States with Q fever studied by Tigertt and Benenson (15) and three patients with Rocky Mountain spotted fever were subsequently included in the present study (Table I).[2]

Selection of capillary bed

Two areas are readily accessible for *in vivo* capillary examination in the human subject—the bulbar conjunctiva and the skin of the nail-fold. Since the vessels of the bulbar conjunctiva are often grossly injected during

[1] This study was sponsored by the Commission on Hemorrhagic Fever and supported in part by the Armed Forces Epidemiological Board by a contract with the Office of the Surgeon General.

[2] The capillary studies pertaining to Q fever were performed in collaboration with Dr. Charles L. Wisseman, Professor of Microbiology, University of Maryland School of Medicine.

many febrile or upper respiratory illnesses, and since preliminary observations indicated minimal nail-fold capillary alterations as a result of fever or respiratory illness *per se*, the nail-fold bed was selected for further capillary studies. Moreover, the utilization of the nail-fold capillary bed ensured that the portion of the minute vascular system selected for study was anatomically and functionally comparable in all patients.

Method of examination

The patients were examined in the recumbent position, with the extended arm at the level of the sternum. Cedar oil was applied to the base of the nail-fold of the fourth finger of the left hand and the terminal capillary loops visualized with a Leitz capillary microscope at a magnification of 80×. As it was impossible to maintain constant room temperatures, two heavy blankets were placed over the patients to induce reflex vasodilatation. No observations were made until the fingers remained constantly warm to the touch.

Criteria of capillary changes

Four indices of nail-fold capillary alterations were followed daily:

1) *Degree of capillary dilatation.* The capillary vessels of the nail-fold are arranged in parallel hairpin shaped loops which vary considerably in length and width. The majority of these loops are not uniform in caliber, but gradually increase in diameter during the transition from arterial to venous segments. Although the walls of the capillary loops are invisible, the absence of any significant marginal plasma zone permits the true diameter to be accurately gauged from the width of the capillary blood stream (16). After study of several microscopic fields comprising a total of approximately 50 to 75 vessels, 8 to 10 representative loops were selected and the widest portions of these vessels (invariably the venous segments) measured with an ocular micrometer. With increased experience, this technique was found to provide a reproducible index of mean widest capillary diameter which generally fell within ± 3 micra of the value obtained by averaging the sum of the widest segments of the individual capillary diameters. Although less sensitive to caliber alterations, such a technique was distinctly more practical than the cinematographic analysis utilized by Crawford (16), wherein individual nail-fold capillary loops were found to vary in diameter from moment to moment, the venous segment changes normally not exceeding ± 1.5 micra.

The widest (venous) segment diameters were utilized for this study since previous observations in approximately three hundred persons with various noninfectious illnesses indicated that such measurements generally reflected the largest increases in capillary diameter during reaction to injury (anoxia, cold, trauma) (17). Comparable observations have previously been reported for Raynaud's disease (18), polycythemia vera (19), and congestive heart failure (20).

TABLE I

Diagnosis in 51 febrile patients with diseases other than hemorrhagic fever

No. of patients	Diagnosis
14	Fever of unknown origin
9	Q fever
6	Acute tonsillitis
5	Acute sinusitis
4	Pneumococcal lobar pneumonia
4	Infectious hepatitis
3	Acute vivax malaria
1	Infectious mononucleosis
1	Generalized urticaria
1	Scrub typhus
3	Rocky Mountain spotted fever

2) *Capillary vasomotor activity.* Intermittent gaps in blood flow normally occur in the nail-fold capillary loops. These gaps in blood flow probably reflect intermittent vasoconstriction of the arterial segments of the capillary loops or of the parent arterioles (21), thus constituting an index of vasomotor activity of the minute vascular bed. Although the gaps in blood flow in any given vessel were irregular and occurred independently of those seen in adjacent capillary vessels, the average number of interruptions in blood flow per minute, as determined in five adjacent vessels, provided a reasonably reproducible index of capillary vasomotor activity. The results of such a study, however, cannot be considered as an absolute indication of capillary vasomotor activity, since the "gaps" in capillary blood flow may not be due to interruptions in blood flow but may also represent changes in arteriolar axial streaming of red blood cells with capillary plasma "skimming."

3) *Rate of capillary blood flow.* With experience it was found that estimates of stagnation, slowed, normal, or accelerated capillary blood flow were consistent in a given patient at a given time and that comparisons between patients or in one patient at different times were valid. Such estimates provided information which was as useful to the present study as were accurately timed measurements.

4) *Sludging.* Normally, red blood cells flow homogeneously through the capillary bed. In some disease states they adhere to each other in the form of irregular globules constituting microscopic emboli known as sludge (22). The intensity of sludging was observed and graded from 0 to 4 plus.

Response to l-norepinephrine

A continuous intravenous infusion of *l*-norepinephrine was given to hemorrhagic fever patients during the phase of hypotension (diastolic arterial pressures under 50 mm. Hg) and to 8 nonhypotensive hemorrhagic fever patients during the first afebrile day. The infusion of *l*-norepinephrine was increased at 10 minute intervals by increments of 0.05 to 0.07 gamma per Kg. per minute. With each increment the response of the nail-fold capillary bed and of the arterial blood pressure was recorded.

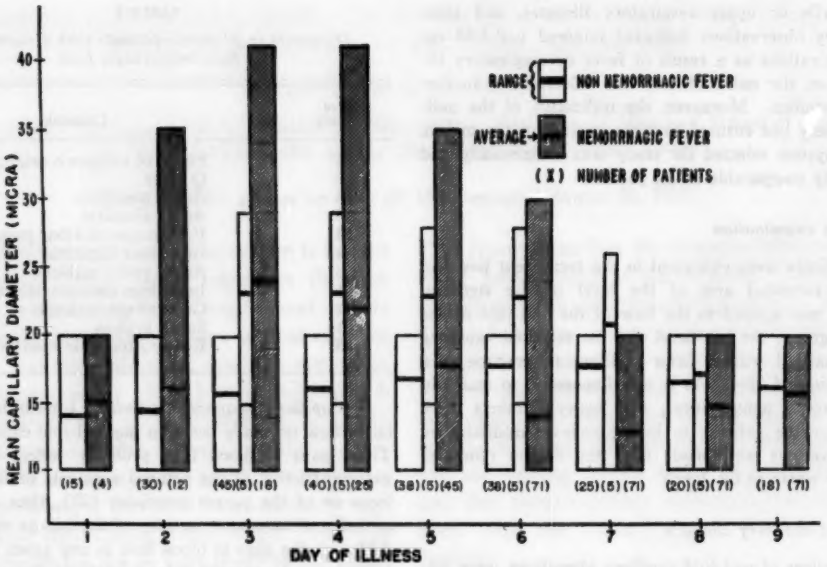

FIG. 1. SERIAL OBSERVATIONS OF MEAN WIDEST NAIL-FOLD CAPILLARY DIAMETER IN PATIENTS WITH HEMORRHAGIC FEVER AND WITH THE OTHER INFECTIOUS ILLNESSES LISTED IN TABLE I

Center column indicates mean widest capillary diameter changes in the patients with Rocky Mountain spotted fever, scrub typhus, and infectious mononucleosis. (The apparent fixation of the lower range of capillary diameter at 10 micra represents those patients with initial values at 10 micra who manifested no nail-fold capillary alterations throughout illness.)

Evaluation of capillary changes

Control baseline nail-fold capillary observations in hemorrhagic fever patients were unobtainable as all patients were clinically ill at the time of admission. Indeed, only twelve patients were examined prior to the third day of illness. Evaluation of the capillary findings during the febrile and hypotensive phases of hemorrhagic fever was therefore based upon 1) serial alterations of the capillary bed as the illness progressed and subsequently during convalescence, and 2) comparison with groups of patients with infectious diseases other than hemorrhagic fever. The previous nail-fold capillary examinations performed in approximately three hundred persons with various noninfectious illnesses (17) served as the background experience for these capillary studies and permitted on over-all evaluation of the vascular changes observed during the present investigation.

RESULTS

I. *Hemorrhagic fever*

A) *Changes observed in the nail-fold capillary bed during the course of hemorrhagic fever*

1) *Capillary dilatation.* The mean widest capillary diameter on the first day of illness of patients with hemorrhagic fever and those with other febrile diseases fell within a range of 10 to 20 micra, with an over-all average of 15 micra. This is comparable to observations by Crawford that the majority of capillary venous segments in the normal nail-fold bed measure 15 to 17 micra in diameter (16). Progressive capillary dilatation involving both the arterial and venular segments of the capillary loops developed in the patients with hemorrhagic fever. Dilatation of the venular segments was always the more pronounced. Increases in mean widest capillary diameter were usually maximum during the third or fourth days of illness (Figure 1). At this stage, seven of the patients (10 per cent) with hemorrhagic fever developed minute hemorrhages in the capillary bed consisting of red blood cells surrounding the length of the vessel or consolidated hemorrhages, usually at the tip of the capillary loop. In the five subjects with hemorrhages surrounding the length of the capillary, the red blood cells were distributed in single or double file and it was postulated that the cells had escaped by a process of diapedesis; in

the two patients with consolidated hemorrhages, an actual rupture of the capillary loops was indicated by cessation of blood flow distal to the hemorrhage with diversion of the blood flow into the hemorrhage site.

By the fifth day of hemorrhagic fever, generalized dilatation of the nail-fold capillary bed had usually begun to subside, though isolated dilatation of one or more venular segments frequently persisted for one to two weeks. These dilatations were particularly striking in their abrupt demarcation from the arterial segments, thereby resembling microaneurisms. In those patients who developed hypotension, dilatation of the nail-fold capillary bed was often marked and extended beyond the fourth day of illness (Figure 2). The capillary dilatation usually persisted throughout the duration of the hypotensive period.

By the sixth day of hemorrhagic fever, capillary constriction appeared, and during the seventh and eighth days of illness, the capillary vessels attained their narrowest caliber. Although this constriction involved the arterial segments of the capillary loops most severely, the venular segments were also attenuated. In patients who developed significant hypertension, the capillary bed became severely constricted, though intense constriction also occurred in the absence of clinical hypertension.

By the ninth or tenth day of illness, the diameters of the arterial and venous capillary segments generally began to return towards normal. Serial photomicrographic tracings of nail-fold capillary diameter changes in a representative patient with a moderately severe case of hemorrhagic fever are shown in Figure 3.

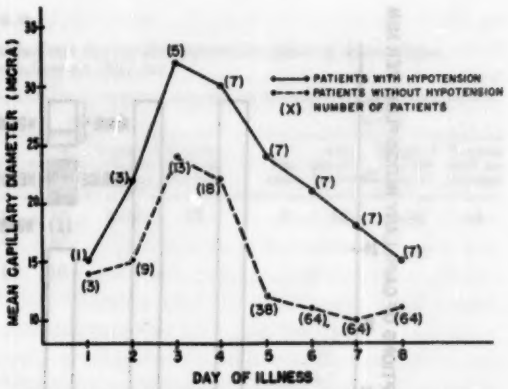

FIG. 2. SERIAL CHANGES IN MEAN WIDEST NAIL-FOLD CAPILLARY DIAMETER IN HEMORRHAGIC FEVER PATIENTS WITH AND WITHOUT CLINICAL EVIDENCE OF HYPOTENSION

2) *Capillary vasomotor activity.* The intensity of capillary vasomotor activity (and/or plasma "skimming") during hemorrhagic fever exhibited an inverse relation to the severity of capillary dilatation. During the initial four days of illness, few

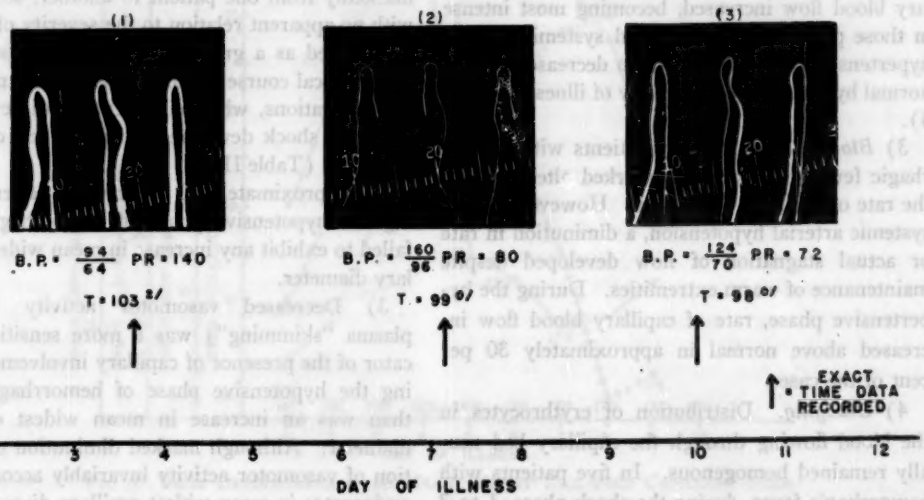

FIG. 3. SERIAL PHOTOMICROGRAPHIC TRACINGS OF THE NAIL-FOLD CAPILLARY BED IN A PATIENT WITH CONFIRMED HEMORRHAGIC FEVER (100 X)

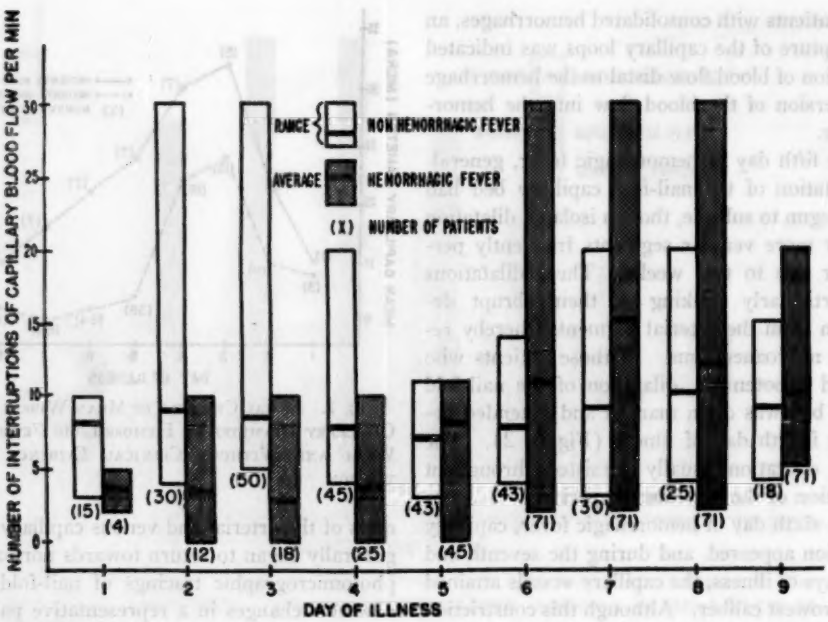

FIG. 4. SERIAL OBSERVATIONS OF NAIL-FOLD CAPILLARY VASOMOTOR ACTIVITY (AND/OR PLASMA "SKIMMING") IN PATIENTS WITH HEMORRHAGIC FEVER AND WITH THE OTHER INFECTIOUS ILLNESSES LISTED IN TABLE I.

"gaps" in capillary blood flow were seen. If systemic arterial hypotension developed, the "gaps" in blood flow usually subsided entirely. By the fifth day of illness, the frequency of "gaps" in capillary blood flow increased, becoming most intense in those patients who developed systemic arterial hypertension, and then began to decrease towards normal by the ninth or tenth day of illness (Figure 4).

3) *Blood flow.* In most patients with hemorrhagic fever there were no marked alterations in the rate of capillary blood flow. However, during systemic arterial hypotension, a diminution in rate or actual stagnation of flow developed despite maintenance of warm extremities. During the hypertensive phase, rate of capillary blood flow increased above normal in approximately 30 per cent of the cases.

4) *Sludging.* Distribution of erythrocytes in the blood flowing through the capillary bed usually remained homogenous. In five patients with hemorrhagic fever, during the shock phase, 1 to 2 plus clumping of red blood cells was seen.

B) *Relation of nail-fold capillary changes to the clinical course of hemorrhagic fever*

1) The degree of capillary dilatation varied markedly from one patient to another, sometimes with no apparent relation to the severity of illness. Considered as a group, however, patients with a mild clinical course exhibited minimal or no capillary alterations, whereas patients with severe illness and shock developed more marked capillary dilatation (Table II).

2) Approximately 30 per cent of patients during the hypotensive phase of hemorrhagic fever failed to exhibit any increase in mean widest capillary diameter.

3) Decreased vasomotor activity (and/or plasma "skimming") was a more sensitive indicator of the presence of capillary involvement during the hypotensive phase of hemorrhagic fever than was an increase in mean widest capillary diameter. Although marked diminution or cessation of vasomotor activity invariably accompanied an increase in mean widest capillary diameter, one third of patients without detectable capillary dila-

TABLE II

Increases in mean widest nail-fold capillary diameter in patients during the hypotensive phase of hemorrhagic fever and during other infectious illnesses

Severity of clinical course	Mild				Moderate				Severe			
Diagnosis	No. cases	Avg. increase (micra)	Range of increase (micra)	% cases with no increase	No. cases	Avg. increase (micra)	Range of increase (micra)	% cases with no increase	No. cases	Avg. increase (micra)	Range of increase (micra)	% cases with no increase
Hemorrhagic fever	10	6	0–20	50	8	10	0–20	37	7	16	0–25	14
Scrub typhus					1	12						
Rocky Mt. spotted fever	2	0			1	10						
Infectious mononucleosis	1	5										
Generalized urticaria following penicillin given for upper respiratory illness	1	10										

tation exhibited a significant reduction in vasomotor activity. Stated another way, during the hypotensive phase of hemorrhagic fever, 70 per cent of the patients exhibited an increase in mean widest capillary diameter, whereas 80 per cent of the patients showed a reduction in vasomotor activity.

4) Subsidence of capillary dilatation or development of capillary constriction occurred at the time the blood urea nitrogen was rising rapidly and albuminuria was maximal (Figure 5).

5) Changes in venous hematocrit seemed to bear a distinct relation to changes in nail-fold capillary dilatation; thus, evidence of greatest plasma leakage as indicated by a rising hematocrit occurred during the phase of maximum capillary dilatation, and greatest resorption of plasma as indicated by a falling hematocrit occurred during maximum

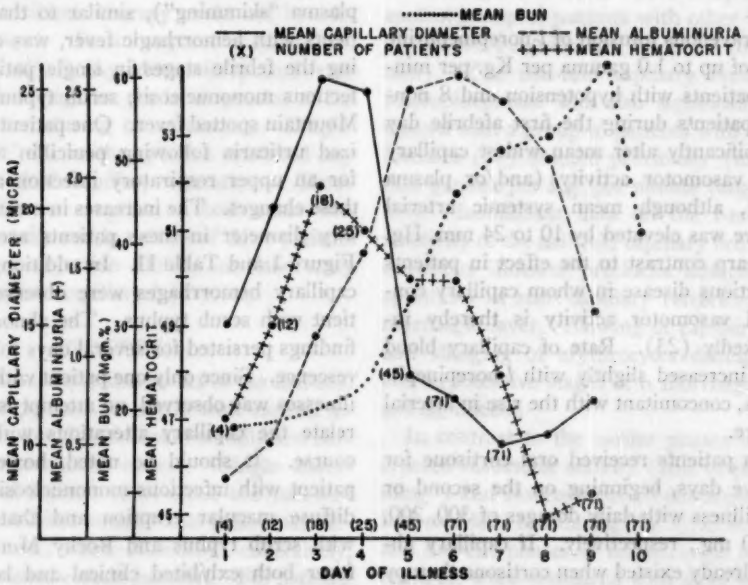
MEAN BUN
MEAN CAPILLARY DIAMETER
(X) NUMBER OF PATIENTS
MEAN ALBUMINURIA
++++MEAN HEMATOCRIT
MEAN CAPILLARY DIAMETER (MICRA)
MEAN ALBUMINURIA (+)
MEAN BUN (Mgm.%)
MEAN HEMATOCRIT
(4) (12) (18) (25) (45) (71) (71) (71) (71) (71)
1 2 3 4 5 6 7 8 9 10
DAY OF ILLNESS

FIG. 5. SERIL DETERMINATIONS OF MEAN WIDEST NAIL-FOLD CAPILLARY DIAMETER, MEAN ALBUMINURIA, MEAN BLOOD UREA NITROGEN AND MEAN VENOUS HEMATOCRIT IN PATIENTS WITH HEMORRHAGIC FEVER

capillary constriction (Figure 5). Since intake of fluids was usually carefully controlled to match total output (10), these hematocrit changes probably reflect alterations in general capillary permeability.

6) Gross conjunctival injection and facial-thoracic flush persisted several days to one or two weeks after subsidence of the nail-fold capillary dilatation and remained unaltered despite the development of nail-fold capillary constriction. The conjunctival injection and facial-thoracic flush were also consistently observed in those patients exhibiting no evidence of nail-fold capillary dilatation or decreased vasomotor activity.

C) *Effect of various forms of therapy on nail-fold capillary alterations in hemorrhagic fever*

1) Tetracyclines in oral dosages of up to 4 Gms. daily did not significantly alter the nail-fold capillary changes.

2) The intravenous infusion of concentrated human albumin into five patients during the shock phase raised systemic arterial blood pressure and accelerated capillary blood flow but did not alter the other characteristics of the dilated nail-fold capillary bed.

3) The intravenous infusion of *l*-norepinephrine in quantities of up to 1.0 gamma per Kg. per minute into 10 patients with hypotension and 8 nonhypotensive patients during the first afebrile day failed to significantly alter mean widest capillary diameter or vasomotor activity (and/or plasma "skimming"), although mean systemic arterial blood pressure was elevated by 10 to 24 mm. Hg. This is in sharp contrast to the effect in patients without infectious disease in whom capillary constriction and vasomotor activity is thereby increased markedly (23). Rate of capillary blood flow usually increased slightly with *l*-norepinephrine infusions, concomitant with the rise in arterial blood pressure.

4) Sixteen patients received oral cortisone for five successive days, beginning on the second or third day of illness with daily dosages of 300, 200, 200, and 100 mg., respectively. If capillary abnormalities already existed when cortisone therapy was initiated, as seen in eight patients, there was no significant restorative effect. Six of the eight patients who received cortisone prior to the appearance of vascular alterations subsequently developed capillary dilatation and impaired vasomotor activity (and/or plasma "skimming").

5) During the phase of maximum capillary dilatation, three patients were given single intravenous doses of 0.6 mg. per Kg. of Benadryl® over a five minute period. Rate of capillary blood flow and vasomotor activity (and/or plasma 'skimming") increased slightly. The mean widest capillary diameter decreased 5 micra in two patients. These changes were transient, persisting 10 to 30 minutes. In the one patient in whom this initial dose was followed by an intravenous infusion of Benadryl® at a rate of 0.3 mg. per Kg. per hour, the initial alteration in the capillary bed was not maintained.

II. *Febrile illnesses other than hemorrhagic fever*

Those febrile illnesses other than hemorrhagic fever that were studied for evidences of capillary alterations are indicated in Table I. Most of these patients, despite pyrexia of up to 105° F., exhibited no consistent detectable nail-fold capillary changes. An increase in mean widest capillary diameter and decreased vasomotion (and/or plasma "skimming"), similar to that seen in patients with hemorrhagic fever, was observed during the febrile stages in single patients with infectious mononucleosis, scrub typhus, and Rocky Mountain spotted fever. One patient with generalized urticaria following penicillin administration for an upper respiratory infection also exhibited these changes. The increases in mean widest capillary diameter in these patients are indicated in Figure 1 and Table II. In addition, several pericapillary hemorrhages were observed in the patient with scrub typhus. The abnormal capillary findings persisted for several days following defervescence. Since only one patient with each of these illnesses was observed, no attempt is made to correlate the capillary alterations with the clinical course. It should be noted, however, that the patient with infectious mononucleosis developed a diffuse macular eruption and that the patients with scrub typhus and Rocky Mountain spotted fever both exhibited clinical and laboratory evidences of diffuse and severe injury to the capillary vascular system. Such capillary injury was indicated by a marked rise in venous hematocrit, pe-

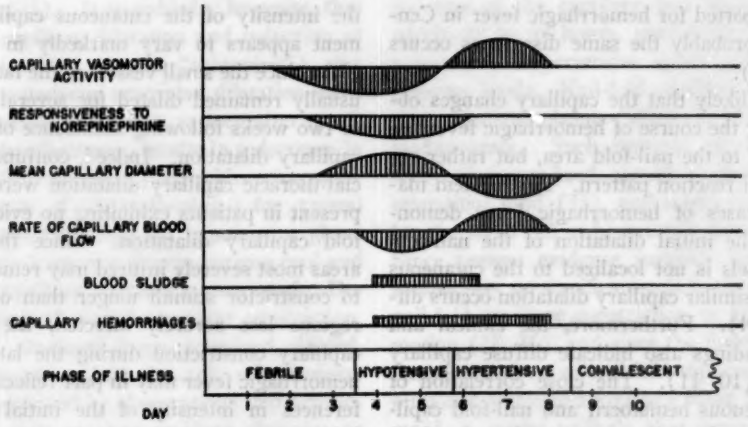

FIG. 6. INTERPRETIVE SUMMARY OF NAIL-FOLD CAPILLARY CHANGES DURING HEMORRHAGIC FEVER

techial and hemorrhagic lesions on cutaneous and mucosal surfaces, strongly positive Rumpel-Leede tests, systemic arterial hypotension, and 2 to 3 plus albuminuria with microscopic hematuria, despite the institution of specific antibiotic therapy after approximately four days of illness. Moreover, the possibility that the severity and duration of the nail-fold capillary alterations may have been mitigated by the antibiotic therapy could not be adequately evaluated. In subsequent studies, two other patients with Rocky Mountain spotted fever were observed throughout the latter portion of the febrile phase and during convalescence. Despite pyrexia to 105° F., neither patient exhibited any significant nail-fold capillary alterations. However, the cutaneous eruption in both cases was minimal in intensity and in neither of these patients was there any objective clinical or laboratory signs of generalized capillary injury.

DISCUSSION

By direct microscopy, alterations of the nail-fold capillary bed in patients acutely ill with various infectious diseases could be followed serially and correlated with the clinical course. Single observations were of limited value since the nail-fold capillary bed exhibited considerable individual variations. However, when considered collectively, the changes assumed certain patterns. For patients with hemorrhagic fever, the capillary alterations are presented as an interpretive summary (Figure 6). Arranged in order of frequency, decreased vasomotor activity (and/or plasma "skimming"), refractoriness to *l*-norepinephrine, dilatation, and hemorrhagic diathesis were the outstanding features observed in the nail-fold capillary bed during the febrile and hypotensive phases of illness. The decrease in vasomotor activity (and/or plasma "skimming"), the capillary dilatation, and the hemorrhagic diathesis, when compared with the control group of patients with other infectious illnesses, were significant ("t" > 3.5 for each alteration). The nail-fold capillary refractoriness to *l*-norepinephrine was also significant when contrasted with a group of patients with noninfectious diseases (23) studied previously (in the 0.16 to 0.20 gamma per Kg. per minute range, "t" equals 5.4). Insufficient data on the *l*-norepinephrine reactivity of the nail-fold capillary bed during other infectious illnesses precludes analysis as to the specificity of such capillary refractoriness for hemorrhagic fever. Slowing of capillary blood flow and sludging of erythrocytes during the febrile and hypotensive stages of hemorrhagic fever were not significant.

In contrast to the earlier phases of illness, increased vasomotor activity (and/or plasma "skimming") and vasoconstriction developed in the nail-fold capillary bed during the hypertensive-oliguric and diuretic phases of hemorrhagic fever. These changes, when compared with those of the control group of patients with other infectious illnesses, were significant ("t" > 2.6 for each alteration). Similar alterations in the nail-fold capillary bed

have been reported for hemorrhagic fever in Central Russia, probably the same disease as occurs in Korea (24).

It appears likely that the capillary changes observed during the course of hemorrhagic fever are not restricted to the nail-fold area, but rather reflect a general reaction pattern. Postmortem material from cases of hemorrhagic fever demonstrates that the initial dilatation of the nail-fold capillary vessels is not localized to the cutaneous area but that similar capillary dilatation occurs diffusely (12–14). Furthermore, the clinical and laboratory findings also indicate diffuse capillary dysfunction (10, 11). The close correlation of changes in venous hematocrit and nail-fold capillary diameter especially suggests that the nail-fold capillary alterations may reflect generalized similar capillary changes. However, Russian investigators have recently demonstrated that the cutaneous capillaries of the chest and abdomen remain dilated during the phase of nail-fold capillary constriction and conclude that the capillary alterations during hemorrhagic fever are of a "segmental character" (24). Such regional dissociation of small vessel caliber during the *later* stages of hemorrhagic fever has been confirmed by the present study. Although the basis for this segmental capillary involvement is as yet undetermined, it seems unlikely that an infectious or humoral agent would constrict the capillary bed in one cutaneous area while simultaneously dilating comparable vessels in another cutaneous area. As will be indicated, it also seems improbable that regional variations in neurogenic factors produce the "segmental" capillary alterations. An alternative explanation which considers the regional variations in intensity of the initial capillary damage appears the most plausible. Although the capillary injury initiated during the febrile and hypotensive phases of hemorrhagic fever is generalized, the severity of this capillary injury is not uniform. Postmortem studies indicate that certain capillary areas such as the renal medulla, anterior pituitary, and right atrium are consistently involved more extensively than are most other areas (12–14, 25). Furthermore, 20 per cent of all patients with hemorrhagic fever exhibited no detectable nail-fold capillary alterations during the febrile and hypotensive phases despite concomitant clinical evidence of generalized capillary dysfunction. Even the intensity of the cutaneous capillary involvement appears to vary markedly in different regions since the small vessels of the face and thorax usually remained dilated for several days to one or two weeks following subsidence of the nail-fold capillary dilatation. Indeed, conjunctival and facial-thoracic capillary dilatation were consistently present in patients exhibiting no evidence of nail-fold capillary dilatation. Since those capillary areas most severely injured may remain refractory to constrictor stimuli longer than other vascular regions less severely affected, the "segmental" capillary constriction during the latter stages of hemorrhagic fever may in part reflect regional differences in intensity of the initial small vessel injury.

The physiologic basis for the nail-fold capillary caliber alterations during hemorrhagic fever is unknown. The initial capillary dilatation might be passive (*i.e.*, increased "stretching") secondary to increased inflow of blood, impairment of outflow, or increased blood volume with "capillary storage" of blood such as described for polycythemia vera (19); or the capillary dilatation might be active due to loss of vascular tone. During the febrile and hypotensive phases of hemorrhagic fever, digital blood flow and antecubital venous pressures are often normal (26–28); blood volume is decreased (29). Such evidence suggests that the nail-fold capillary dilatation is related to loss of vascular tone, although the participation of venular constriction cannot be excluded. Similarly, the nail-fold capillary attenuation might be passive (*i.e.*, diminished "stretching") secondary to decreased inflow of blood, facilitation of outflow, or decreased blood volume (antithesis of the "storage effect"); or the capillary attenuation might be active due to increased vascular tone. During the hypertensive phase of hemorrhagic fever, digital blood flow may be increased above normal (26, 27); blood volume and antecubital venous pressures are generally normal (28, 29). Such evidence suggests that the nail-fold capillary attenuation results from an active increase in vascular tone.

The mechanism underlying the generalized capillary injury initiated early in the course of hemorrhagic fever is undetermined. It has been postulated that intense and generalized arteriolar dilatation *per se* might induce the diffuse capil-

lary damage (25). It is unlikely, however, that the nail-fold capillary dilatation and reduction of vasomotion (and/or plasma "skimming") was secondary to cutaneous arteriolar dilatation since cutaneous arteriolar dilatation, as seen after cervicothoracic sympathectomy, results in a decrease in nail-fold capillary diameter (18). Moreover, during the infusion of *l*-norepinephrine for several days in 10 patients during the hypotensive phase of hemorrhagic fever, the fingers became cool and the nail-fold capillary circulation decreased. Despite these signs of digital arteriolar constriction, the nail-fold capillary loops remained dilated. Further evidence against the role of arteriolar dilatation is provided by observations that arteriolar dilatation fails to alter capillary diameter or vasomotor activity of the normal mammalian capillary bed (18, 30, 31). Indeed, even the existence of generalized arteriolar dilatation in the usual patient with hemorrhagic fever seems improbable, since a widening of arterial pulse pressure, a decrease in peripheral resistance, and "capillary pulsations" were generally absent (9–11, 32). It appears more likely, therefore, that unidentified noxious factors act directly upon the capillary bed during the febrile and hypotensive phases of hemorrhagic fever. Attempts to define the presence of humoral capillary damaging factors in patients with hemorrhagic fever will be presented in a subsequent paper.

The etiology of the hypotensive phase of hemorrhagic fever has received considerable study. A generalized loss of arteriolar tone, or inability of the arterioles to respond to constrictor stimuli, suggested as a possible causative mechanism (25), did not appear responsible as peripheral resistance was found elevated in most patients with hypotension (32). Generalized capillary dilatation, however, may induce arterial hypotension by reducing effective circulating blood volume (33, 34). A further reduction of effective circulating blood volume, induced by plasma leakage through damaged capillary walls, would be favored by a decrease in capillary vasomotor activity (35). Thus, two mechanisms for the systemic arterial hypotension during hemorrhagic fever—capillary dilatation and loss of vasomotor activity—are reflected in the changes seen in the nail-fold capillary bed.

Compared to the development of nail-fold capillary dilatation over a 48 to 96 hour period, constriction of the capillary bed usually developed within 24 to 48 hours, paralleling the rapid appearance of hypertensive systemic arterial blood pressure levels. Nail-fold capillary vasomotor activity (and/or plasma "skimming") increased simultaneously. Such increase of vasomotor activity, if generalized, would favor resorption of interstitial fluid (35), and might account in part for the rapid fall in hematocrit during this phase. Since present evidence suggests that neurogenic factors play no significant role in the production of nail-fold capillary constriction or enhanced vasomotion (18, 36), and since the fingers of patients during the phase of nail-fold capillary constriction remained warm and capillary blood flow remained rapid, it seems unlikely that neurogenic factors were primarily responsible for the nail-fold capillary constriction. The role of humoral factors in the production of the nail-fold capillary constriction, however, is unknown. Although the hypertensive state and the nail-fold capillary constriction usually developed at the time albuminuria was maximum and the blood urea nitrogen was increasing rapidly, the participation of renal vasoconstrictor substances remains speculative and unproven.

An agent that mitigates the diffuse capillary damage in hemorrhagic fever has not been found. The tetracyclines, Benadryl®, cortisone, concentrated serum albumin, and *l*-norepinephrine did not significantly prevent or reverse the nail-fold capillary dilatation or loss of vasomotor activity (and/or plasma "skimming"). These agents, moreover, failed to materially alter the clinical course of the illness (11, 37, 38).

The majority of patients acutely ill with infectious diseases other than hemorrhagic fever, despite pyrexia to 105° F., failed to exhibit any significant nail-fold capillary alterations. However, in single patients with scrub typhus and infectious mononucleosis, the serial nail-fold capillary findings during the febrile stages were indistinguishable from those during the febrile and hypotensive phases of hemorrhagic fever. Comparable capillary changes also developed in one patient with Rocky Mountain spotted fever who exhibited concomitant clinical evidence of severe and generalized small vessel injury. The nail-fold capillary alterations during the febrile and hypotensive phases of hemorrhagic fever are therefore not specific, but

appear to represent a basic vascular response pattern to a variety of infectious agents. Whereas the mechanism for these capillary changes during hemorrhagic fever remain unknown, such alterations during scrub typhus and Rocky Mountain spotted fever may be explained, in part at least, by the direct rickettsial invasion of the vessel wall (7). As with hemorrhagic fever, it seems likely that the capillary alterations in the patients with scrub typhus and Rocky Mountain spotted fever are not peculiar to the nail-fold area but reflect the diffuse small vessel injury which characterizes most rickettsial infections (7). Although Q fever is classified as a rickettsial infection, clinically and pathologically this disease is very different from the other rickettsial infections. In man, no important lesions are found outside the lungs; the characteristic diffuse involvement of small blood vessels, including those of the skin such as occurs with the other rickettsial agents, are conspicuously absent (7). It appears significant, therefore, that no consistent nail-fold capillary alterations were noted in the nine patients clinically ill with Q fever. However, since these patients all received specific antibiotic therapy after the first day of fever (15), the possibility of capillary alterations occurring later in the untreated disease cannot be excluded. The significance of the nail-fold capillary changes in the patient with infectious mononucleosis is unknown. Although petechial hemorrhages may appear on the hard palate (39), the presence of the cutaneous eruption and the absence of any pathological description or clinical evidence of visceral capillary injury in this disease (40–42) suggest that the capillary changes in this patient may have been limited to the cutaneous area.

SUMMARY

Alterations of the nail-fold capillary vessels of 71 patients with hemorrhagic fever and 51 patients with a variety of other infectious illnesses have been followed serially by direct microscopy:

A) *Hemorrhagic fever*

1. Arranged in order of frequency, decreased vasomotor activity (and/or plasma "skimming"), refractoriness to *l*-norepinephrine, increase in mean widest diameter, and hemorrhagic diathesis were the outstanding features observed during the febrile and hypotensive phases. Slowing of capillary blood flow and sludging of erythrocytes were less conspicuous.

2. The intensity of the capillary dilatation and loss of vasomotor activity (and/or plasma "skimming") paralleled the severity of the clinical course, although considerable variation occurred in individual cases. Twenty per cent of the patients failed to exhibit any such capillary alterations in the nail-fold area despite concomitant clinical and laboratory evidences of diffuse capillary injury.

3. The dilatation and loss of vasomotor activity (and/or plasma "skimming") in the nail-fold capillary bed were not prevented nor significantly reversed by administration of tetracyclines, Benadryl®, cortisone, serum albumin, or *l*-norepinephrine.

4. The mean widest capillary diameter returned toward normal, in the average patient, by the fifth day of illness. Constriction of the nail-fold capillary bed and heightened vasomotor activity (and/or plasma "skimming") subsequently appeared during the hypertensive-oliguric and diuretic phases of illness.

5. The alterations of the capillary vessels are not peculiar to the nail-fold area; rather they appear to reflect the presence of injurious factors acting diffusely and directly, although not uniformly, upon the capillary vascular system.

B) *Other infectious illnesses*

1. Most patients with infectious illnesses other than hemorrhagic fever, despite pyrexia to 105° F., exhibited no nail-fold capillary alterations.

2. The nail-fold capillary changes in two patients with clinical evidence of diffuse and severe capillary injury during scrub typhus and Rocky Mountain spotted fever were indistinguishable from those seen during the febrile and hypotensive phases of hemorrhagic fever. Comparable alterations were also noted in one patient with infectious mononucleosis associated with cutaneous manifestations.

3. Nine patients with early Q fever, in contrast to the patients with the other rickettsial diseases studied, exhibited no significant alterations of the nail-fold capillary vessels. These differences may be related to differences in the clinical

and pathological findings in the various rickettsial infections.

4. Of the nail-fold capillary changes observed during the febrile and hypotensive phases of hemorrhagic fever and during the course of the other infectious illnesses, none were specific for any given disease; indeed such changes appear to represent a general response pattern to a number of infectious agents that act primarily upon the capillary vascular system. Nail-fold capillary constriction and heightened vasomotor activity (and/or plasma "skimming"), however, as occurred during the hypertensive phase of hemorrhagic fever, appeared to be specific for this infectious illness.

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PLASMA ADRENOCORTICOTROPHIC HORMONE IN ADDISON'S DISEASE AND ITS MODIFICATION BY THE ADMINISTRATION OF ADRENAL STEROIDS

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The depressant effect of adrenal steroids on the release of pituitary adrenocorticotrophic hormone (ACTH) was first demonstrated by Ingle in the experimental animal in 1937 (1). This observation has been confirmed by many investigators (2–7), but not by all (8). In man the clinical, morphological and biochemical evidence for steroidal inhibition of pituitary secretion is impressive but there are few actual measurements of blood ACTH reported. There is substantial evidence for an increased level of circulating ACTH in Addison's disease (9, 10) and postoperative bilaterally adrenalectomized patients (11), but an adequate quantitative evaluation of this activity has not been available. Evidence for an increased secretion of ACTH in the adreno-genital syndrome (12) is also convincing, but similar increases reported in rheumatic fever (13), Cushing's disease (14), pregnancy (14), the early post-partum period (15), and other conditions (16) have not been confirmed.

The present study was undertaken to determine the level of ACTH in peripheral blood in Addison's disease and to observe the effects of adrenal steroids on the alteration of this activity.

METHODS

The assay procedure followed in these studies has been previously described by Nelson and Hume (17) and utilizes the production of corticosteroids by the adrenal cortex of the hypophysectomized dog as a measure of ACTH activity in administered human plasma. It was performed as follows: 40 to 60 milliliters of blood were withdrawn from the patient into a heparinized syringe. The plasma was immediately separated by centrifugation and frozen. Within one to six days the plasma was injected into the assay animal. The assay was performed in a hypophysectomized dog in which a catheter had been placed in the right adrenal vein in such a way as to make intermittent, complete collections of the adrenal venous effluent possible. Physiological saline solution containing 0.01 N hydrochloric acid and 1 mU of U.S.P. Standard ACTH per ml. was used as a reference against which the plasma activity was measured. One, 2, 5 and 8 ml. of this standard, and 20 to 30 ml. of plasma were injected into the adrenal by the femoral vein during a two minute period. (The difference in volumes injected did not appear to affect the result significantly. Although use of whole plasma allows possibly toxic substances to be injected with the ACTH, acid-acetone, and oxycellulose purified extracts of duplicate samples of plasma always showed less ACTH activity than was present in the whole plasma.) One minute later the adrenal venous collection was begun and continued for exactly 10 minutes. A 5 ml. aliquot was removed and the remaining blood was returned to the venous circulation of the dog. The interval between collections was 30 minutes. In the performance of a typical assay, two control samples of 0.01 N HCl in saline and five unknown plasma samples were alternated with nine ACTH standard injections. Studies in this laboratory (18) and by Granirer (15) have shown that ACTH will retain its potency in frozen plasma for at least three or four weeks. The content of 17-hydroxycorticosteroids in the aliquot of whole blood removed from the adrenal vein of the dog was determined by a slight modification of the method of Nelson and Samuels (19).

Each determination required the withdrawal of at least 40 ml. of blood from the patient and it was therefore usually impossible to perform the determinations for ACTH in duplicate. Values of ACTH in this paper are expressed in mU per 100 ml. of plasma. The ACTH concentration of the plasma sample and its standard error were estimated in logarithmic terms by the use of the usual formulae from a comparison of the effect of an injected plasma sample on the release of 17-hydroxycorticosteroids with the response given by graded doses of U.S.P. Standard ACTH (20). By conversion to antilogarithms, the desired values are obtained in mU. Since the addition of logarithms implies multiplication of antilogarithms, the plus-minus symbols (±) usually used for standard errors are changed to the multiplication-division symbols $\left(\frac{\times}{\div}\right)$ for the mU (antilogarithms), as shown in

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[2] Investigator, Howard Hughes Medical Institute.

the following example:

	Estimate of potency	Standard error
In logs	0.903	±0.114
In antilogs	8 mU	×1.30 ÷

In this example, the standard error may also be cited as 30 per cent of the potency estimate: (1.30 − 1.00) 100 equals 30 per cent. Squaring the antilogarithm of the standard error prior to multiplication and division will give the approximate 95 per cent confidence limits of each value. Differences between groups of values were evaluated by Student's "t" test; the two tail test being applied to the differences between groups of values in different patients and the one tail test for differences in the same patient (21). The lower limit of sensitivity of the assay varies somewhat depending on the individual responsiveness of the adrenal gland of the assay animal. In general, a significant increase in 17-hydroxycorticosteroid content of the adrenal venous blood was seen with 0.75 to 1.5 mU of ACTH. Therefore, depending on the amount of plasma administered, the minimal detectable level expressed per 100 ml. of plasma is about 2 to 5 mU per 100 ml. and values shown in the tables as "zero" could thus be somewhat elevated over normal if they should fall below this lower limit of sensitivity. Such values are more correctly expressed as "nondetectable" than as zero or negative values, but for convenience the zero symbol is retained in the tables. It was impossible to calculate satisfactorily an exact upper limit of these zero values.

TABLE I

Plasma ACTH in normal subjects, hospitalized patients with normal adrenal function, and patients with Addison's disease

Patient	ACTH *mU/100 ml.*	× and ÷ by antilog. S.E.M.	Patient	ACTH *mU/100 ml.*	× and ÷ by antilog. S.E.M.
Normal subjects					
J. B.	0	*	D. M.	0	
J. F.	0		N. McW.	0	
D. N.	0		E. R.	0	
D. S.	0		R. F.	0	
Hospitalized patients					
R. A.	0		H. B.	0	
M. S.	0		A. P.	0	
M. U.	0		J. G.	0	
E. U.	0		R. I.	0	
Addisonian patients					
N. P.	7.5	2.44	J. G.	18.1	2.88
E. V.	2.9	1.29	A. R.	6.5	1.84
G. R.	0	*	D. McG.	10.2	1.44
D. R.	0		D. McG.	7.2	1.99
D. R.	10.2	1.43	D. McG.	6.0	1.66
W. L.	9.9	1.52	A. K.†	6.9	2.04
W. L.	14.1	1.46	A. K.	8.1	1.82
W. L.	30.0	1.39	A. K.	5.7	1.87
M. K.	12.4	1.87	W. D.	0	
M. K.	8.6	1.58	W. D.	3.5	1.07
M. K.	20.9	1.57	K. H.	0	
J. H.†	0		L. W.	12.5	1.55
J. H.	0		L. W.	24.8	1.99
J. H.	0		L. W.	26.9	2.02
J. H.	0		C. M.	16.6	2.31
J. C.	0		C. G.	7.2	1.56
J. C.	0		W. G.	18.0	1.74
L. H.	2.6	1.98	W. G.	19.1	1.63
L. H.	0		W. G.	17.1	1.41
E. C.	4.0	1.85	P. T.†	11.3	1.14
M. C.	16.7	2.35	J. S.	3.7	2.20
H. W.	0		G. S.	5.0	1.22
H. McC.	5.3	1.69	E. M.	6.1	1.38
H. McC.	6.7	1.34	J. O'S.	0	
H. McC.	3.3	1.37	M. L.	7.9	1.65
R. S.	24.7	1.58	L. L.	10.7	1.54
S. B.	6.8	2.02	L. L.	6.5	1.56

* Limits cannot be calculated for these values.
† Adrenal insufficiency due to bilateral adrenalectomy.

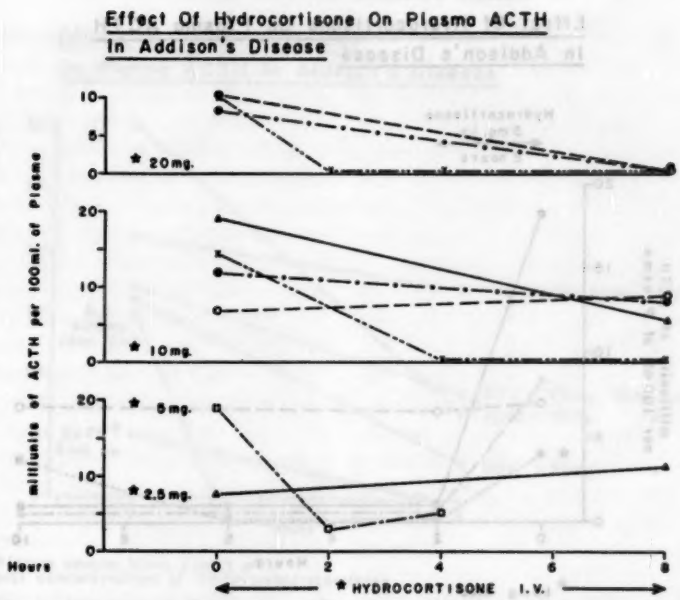

FIG. 1. INFUSION OF HYDROCORTISONE FOR EIGHT HOURS ON EIGHT OCCASIONS TO FOUR ADDISONIAN PATIENTS

The 5 mg. infusion was for four hours in a fifth patient. A distinctive line and symbol is shown for each patient, the symbol indicating the plasma level of ACTH expressed in mU per 100 ml. of plasma. Values at the zero line indicate activity was not detectable. Comparison of initial and final values by one tail "t" test gives a p of < 0.0025 for 20 mg. dose and < 0.20 for 10.0 mg. dose.

RESULTS

Table 1 shows the results of ACTH determinations performed on plasma samples from 29 patients with Addison's disease, three bilaterally adrenalectomized patients, eight hospitalized patients without adrenal dysfunction, and eight normal subjects. All samples were withdrawn in the fasting state between 7:30 and 9:00 A.M. The patients with adrenal insufficiency received no cortisone for at least 24 hours prior to the venous collection and most were on monthly injections of 25 to 50 mg. of desoxycorticosterone trimethylacetate. A few patients were given added salt during the period of study.

In agreement with most investigators, although in disagreement with some, no measurable level of ACTH was found in the normal subjects as well as in the hospitalized patients with no adrenal disease (12, 22, 23). In contrast to this group, only 2 of 28 patients with adrenal insufficiency failed to have ACTH activity if more than one plasma sample were assayed. The ACTH value for the 32 patients with adrenal insufficiency was found to be 8.4 ± 7.4 (S.D.) mU per 100 ml. This value was significantly different from the group of control subjects ($p < 0.001$). There were nine patients with Addison's disease in whom ACTH was not initially detectable. In three of these, repeat values showed detectable levels of activity, in four no repeat determinations could be obtained, and in two repetition gave a negative result on two and four occasions, respectively. One patient who had partial adrenal insufficiency had ACTH activity present on the three occasions on which it was measured.

Figures 1, 2, and 3 illustrate the ACTH values obtained in 10 patients with Addison's disease given 16 infusions of 2.5 to 20 mg. of free hydrocortisone in normal saline for periods varying from two minutes to eight hours. Samples of plasma for ACTH assay were obtained at intervals prior to, during, and up to 12 hours following the

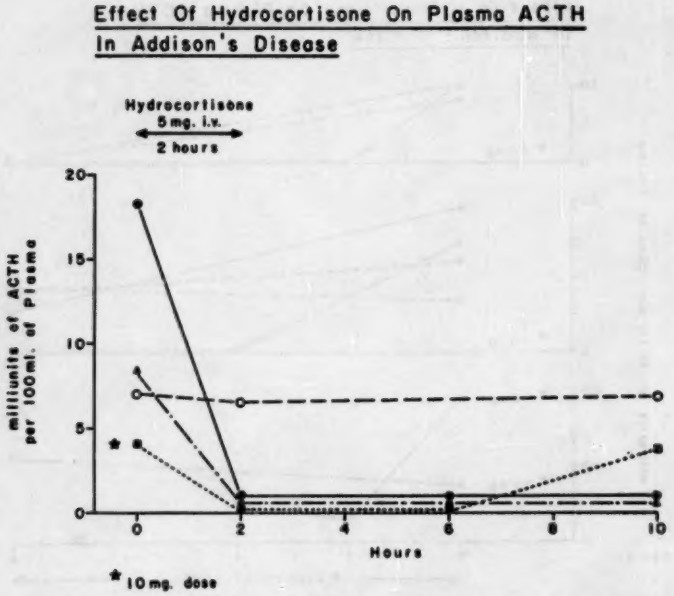

FIG. 2. INFUSION OF 5 MG. OF HYDROCORTISONE OVER A TWO HOUR PERIOD TO THREE PATIENTS AND 10 MG. TO A FOURTH PATIENT

Plasma was assayed up to eight hours following the end of the infusion. P for significance of difference between initial and lowest values is < 0.10.

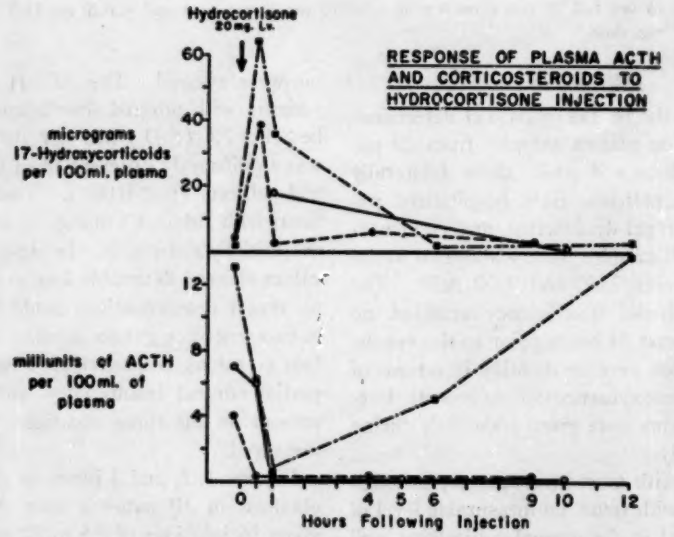

FIG. 3. TWENTY MG. OF HYDROCORTISONE GIVEN INTRAVENOUSLY TO THREE ADDISONIANS IN A TWO MINUTE PERIOD

Blood corticosteroids and ACTH activity were measured concurrently up to 12 hours following this infusion. P for significance of difference between initial and final values at one hour is < 0.10.

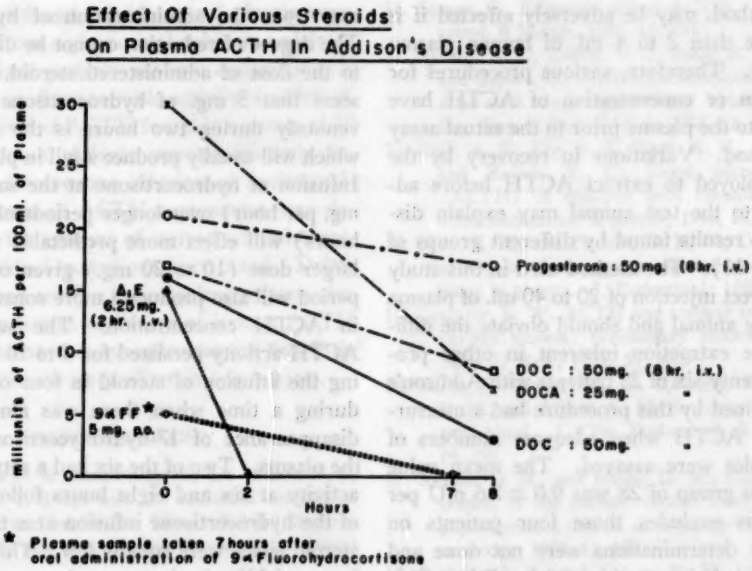

FIG. 4. INFUSION OF PROGESTERONE, FREE DESOXYCORTICOSTERONE (DOC), AND DESOXYCORTICOSTERONE ACETATE (DOCA) IN FOUR ADDISONIANS

Prednisone (Δ1E) was given intravenously in two hours and 9-α fluorohydrocortisone (9α FF) was given by mouth. P for significance of difference between initial and final values for DOC and DOCA is < 0.05.

beginning of the infusion. Plasma 17-hydroxycorticosteroids were determined at the same time as ACTH in three of these studies as shown in Figure 3. The infusions were maintained at a constant rate and, therefore, samples taken at intermediate points represent the effect of the fraction of the total steroid dose administered at that particular time. For example, one-fourth of the 20 mg. dose shown in Figure 1 had suppressed ACTH activity and represents the effect of 5 mg. given for two hours at the same infusion rate. Analysis of the ACTH values obtained prior to and at the lowest point during or following the infusion of hydrocortisone in the studies at 5, 10, and 20 mg. dose levels reveals that a significant fall in ACTH concentration had occurred ($p < 0.05 > 0.025$). Further analysis of the smaller groups separated by dosage and duration of infusion is given in the legend of each table. The groups in these smaller dose ranges were too small to lend significance to observed changes; however, it would appear that the smaller doses were less effective in suppressing ACTH than was the 20 mg. dose. The effect of intravenous desoxycorticosterone, prednisone, and oral 9-α fluorohydrocortisone on the suppression of plasma ACTH are shown in Figure 4.

DISCUSSION

Although attempts have been made to measure the levels of ACTH found in the plasma of patients with Addison's disease, the majority of reports deal only with the qualitative presence or absence of activity. Sydnor, Sayers, Brown, and Tyler have reported values of 2 to 4 mU ACTH per 100 ml. of whole blood in two Addisonian patients (24). Parrott has reported a similar patient with a level of 104 mU ACTH per 100 ml. of plasma while on treatment with 25 mg. of cortison (10). Values for circulating ACTH reported previously by Parrott for normal subjects, and particularly those found in patients with various diseases, have been criticized because they seemed unreasonably high (11). The methods used by these authors and others require the injection of at least 20 ml. of human plasma or its equivalent into the rat for a reliable estimation of ACTH potency in the administered plasma or plasma extract. The rat, the assay animal used in the ascorbic acid

depletion method, may be adversely affected if it receives more than 2 to 4 ml. of human plasma intravenously. Therefore, various procedures for the extraction or concentration of ACTH have been applied to the plasma prior to the actual assay by that method. Variations in recovery by the methods employed to extract ACTH before administration to the test animal may explain discrepancies in results found by different groups of workers (25, 11). The method used in this study allows the direct injection of 20 to 40 ml. of plasma into the assay animal and should obviate the difficulties in the extraction inherent in other procedures. Twenty-six of 28 patients with Addison's disease examined by this procedure had a measurable level of ACTH when adequate numbers of plasma samples were assayed. The mean value found for this group of 28 was 9.0 ± 7.6 mU per 100 ml. (this excludes those four patients on whom repeat determinations were not done and which if included give a value of 8.4 ± 7.4). This value is significantly different ($p < 0.001$) from the results found in the control group of 16 persons. A similar range of activity has been found in two previously reported patients (24) and in the adrenalectomized rat (26).

It has not been possible to correlate the presence or absence of ACTH activity with the degree of pigmentation or with the duration of the disease. However, the fact that all but one of the patients had been on cortisone treatment prior to the study would perhaps minimize differences which would have been otherwise apparent since it has been shown that steroid therapy decreases skin pigmentation in this disease (27). It was of interest that one of the patients with partial adrenal insufficiency had a level of ACTH comparable to the group as a whole. This patient had pigmentation typical of Addison's disease as did all the patients in this group of 32. Van Buchem and Arends have recently reported two patients with Addison's disease without pigmentation in whom they were unable to demonstrate the presence of either ACTH or melanophore stimulating hormone activity (28).

The depressant effect of adrenal steroids on plasma ACTH has been shown in the rat, the adreno-genital syndrome, and in the postoperative adrenalectomized patient. The present studies have shown that a similar reduction in plasma ACTH occurs in the human with Addison's disease upon the administration of hydrocortisone. The degree of reduction cannot be directly related to the dose of administered steroid, but it would seem that 5 mg. of hydrocortisone given intravenously during two hours is the smallest dose which will usually produce a fall in plasma ACTH. Infusion of hydrocortisone at the same rate (2.5 mg. per hour) over longer periods of time (4 to 8 hours) will effect more predictable results, and a larger dose (10 to 20 mg.) given over a shorter period will also produce a more constant reduction in ACTH concentration. The suppression of ACTH activity persisted for 8 to 10 hours following the infusion of steroid in four of six patients during a time when there was almost complete disappearance of 17-hydroxycorticosteroids from the plasma. Two of the six had a return of ACTH activity at six and eight hours following the end of the hydrocortisone infusion at a time when the steroid levels were equally low. There appears to be a variability in the responsiveness of the ACTH regulating mechanism in regard to both the ease of suppression and the rapidity of reappearance of plasma activity following suppression. Factors other than the *plasma* level of 17-hydroxycorticosteroids probably are required to account for the differences noted in the behavior of plasma ACTH in these patients.

Most steroids which have a hydrocortisone-like activity suppress pituitary ACTH to varying degrees and in general this has been shown to correlate with their carbohydrate regulating activity rather than with their salt retaining effects (4). The relative ACTH suppressing activity of progesterone and desoxycorticosterone found in this study in man are in approximate agreement with those observed in the rat (4). The relatively potent synthetic steroids 9-α fluorohydrocortisone and prednisolone also appeared to be inhibitors of ACTH secretion in man.

SUMMARY

A bioassay utilizing the adrenal venous blood content of 17-hydroxycorticosteroids in the hypophysectomized dog as a measure of ACTH activity has been applied to a study of the ACTH content of human plasma. Fifty-one determinations in 32 patients with adrenal insufficiency revealed a mean activity of 8.4 ± 7.4 mU ACTH per 100 ml. of plasma. This was significantly ele-

vated over that found for 16 control plasma samples obtained from eight normal subjects and from eight hospitalized patients without endocrine disease in whom no demonstrable activity was found. The elevated level of ACTH found in a group of 10 of the Addisonian patients was significantly reduced by the intravenous infusion of hydrocortisone. The extent of ACTH suppression was dependent upon the amount of steroid administered, although individual exceptions were noted. Delta-1-hydrocortisone and 9-α fluorohydrocortisone also appeared to be potent inhibitors of human ACTH secretion, whereas desoxycorticosterone had little and progesterone no such effect in one subject.

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THE EFFECT OF PULMONARY VASCULAR PRESSURES ON THE MECHANICAL PROPERTIES OF THE LUNGS OF ANESTHETIZED DOGS [1]

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Since the experiments of von Basch (1) it has been recognized that pulmonary vascular congestion influences the mechanical behavior of the lungs. A number of studies in patients with chronic congestive failure have demonstrated marked changes in pulmonary elasticity (2–6). It has not been possible to determine in such patients the relative contributions of pulmonary edema, other parenchymal changes, and vascular congestion *per se* to the observed changes. Experiments with acute congestion in man or living animals (7–10) and isolated lungs (11) have yielded conflicting results.

The purpose of the present study was to examine in the living animal the effects of acute changes in pulmonary arterial and venous pressures and blood flow on the mechanical behavior of the lungs. The left atrial pressure and the pulmonary blood flow were varied independently. In one set of experiments, volume-pressure curves were obtained during stepwise inflation and deflation of the lung over a wide range of volume starting from the passively collapsed state. In another group, pulmonary compliance and flow-resistance were studied during continuous cycling of the lungs in the normal tidal range of lung volume.

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METHOD

All experiments were done in open-chest dogs. The dogs, weighing 16 to 26 Kg., were anesthetized with Nembutal® (25 to 45 mg. per Kg. body weight, intravenously). The chest was opened by splitting the sternum, and the chest wall was retracted to minimize crowding of the intrathoracic viscera during inflation of the lungs. The right ventricle was replaced by a pump without interruption of the circulation as in an experimental preparation previously described (12). Systemic venous blood was diverted into a reservoir from which it was pumped into either the main pulmonary artery or into the two pulmonary artery branches separately.

When both pulmonary arteries were cannulated, the blood flow in the lung to be studied was varied by changing distribution of flow. In addition, pulmonary flow could be varied by changing pump output. Left atrial pressure was varied by mechanically altering the systemic arterial resistance (constriction of the ascending aorta, inflation of balloon in ascending aorta, systemic A-V shunt). Sometimes additional adjustment of left atrial pressure was obtained with an overflow system between the left atrium and the venous reservoir. In this way changes could be made in pulmonary flow or left atrial pressure independent of each other.

Pulmonary blood flows were measured with rotameters. Pressures in the pulmonary arteries distal to the respective cannulae, in the left atrium, and in the femoral or carotid arteries were measured with electromanometers. These values were recorded on a direct-writing Sanborn oscillograph.

Two methods were used to measure the mechanical characteristics of the lung.

1. *Stepwise inflation and deflation of one lung (eight dogs).* In this group of experiments, the blood flow to each lung was measured and could be varied independently. The trachea was divided by means of a G. Wright double-lumen cannula (13), which afforded air-tight separation of the two lungs. Airway pressures were recorded with an inductance manometer from points near the carina. The lung under study was connected to a closed system filled with a gas mixture which had approximately the same initial oxygen and carbon dioxide tensions as mixed venous blood; the gas exchange in this lung was thereby minimized. The other lung was ventilated with 30 per cent oxygen by a Starling pump.

A hand-operated Starling pump was interposed between the test lung and a spirometer. The lung was allowed to deflate passively and was then inflated in steps (50 to 100 ml.) to an airway pressure of 30 to 40 cm. H_2O (see Figure 1). Deflation proceeded in similar steps; when a pressure near atmospheric was reached, the lungs were allowed to deflate passively into the spirometer. Transpulmonary pressure decreased in the intervals between successive volume steps during inflation; to a lesser extent, the reverse obtained during deflation. Because of this time dependence, it was necessary to follow a fixed time schedule for inflation and deflation. Each volume step had the same duration in each run. The interval between steps was varied in different experiments from 2 to 10 seconds. The interval between runs was one to five minutes, but constant during each experiment. The total time per run ranged from less than one minute to four minutes.

The volume returned to the spirometer on deflation was always less than the volume introduced on inflation. This deficit was greatest on the first cycle and appeared to result from air trapping within the lungs. After a few cycles the volume deficit reached a minimum value and remained constant (4 to 10 per cent of the inflation volume). This volume deficit was assumed to be due to a slight leakage.

During the inflation, the arterial pressure in the inflated lung rose due to the increased vascular resistance during inflation (Figure 1). In most of the experiments, the flow through that lung also decreased, in some runs up to 40 per cent; this could be avoided by adjusting screw clamps on the inflow tubings. In several experiments, the recorded left atrial pressure also increased slightly, probably due to elevation of the heart.

2. *Continuous cycling of the lung volume over the normal tidal range (four dogs).* In these experiments the main pulmonary artery was perfused and the airway was not divided. The animal was enclosed in a body plethysmograph. The tubes connecting the vascular system to the blood pump, and the trachea to the respiratory pump, ran through air-tight seals in the plethysmograph wall. The respiratory pump consisted of a blower with an electrically controlled valve, and was connected to the trachea. Versatile control of mean pressure, pressure amplitude, and cycling frequency was possible. The pressure pattern was approximately sinusoidal. End-expiratory carbon dioxide was continuously recorded with a Liston Becker carbon dioxide analyzer and maintained at desired level by adjustment of the tidal volume. No arterial hypoxia occurred. Lung volume changes were measured with an electrically recording Krogh spirometer connected to the body chamber. Transpulmonary pressure versus lung volume change and/or pressure versus flow rate were displayed on an oscilloscope.

Pulmonary compliance was measured as the ratio of the tidal volume to the change in transpulmonary pressure between the instants of zero air flow at the tidal volume extremes. Pulmonary flow-resistance, which includes air flow-resistance and tissue viscosity, was de-

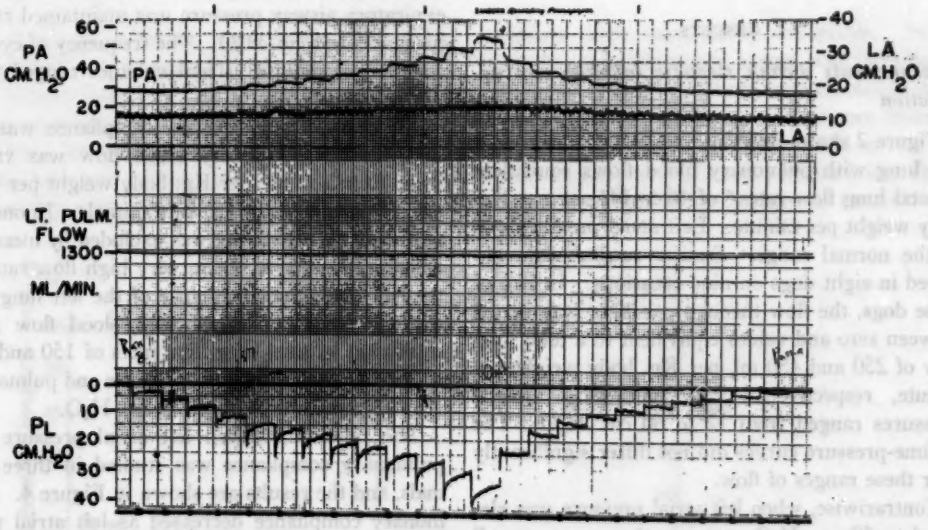

FIG. 1. EFFECTS OF STEPWISE INFLATION AND DEFLATION OF LEFT LUNG ON VASCULAR AND AIRWAY PRESSURES IN THE LUNG (EXPERIMENT NO. 62)

PA refers to left pulmonary artery pressure; LA refers to left atrial pressure; P_L refers to left airway pressure. Volume increments equal 95 ml. Each volume step was done in less than one-half second. There were 10 second intervals between each step. In this experiment, both pulmonary artery inflow tubings were constricted so that the changes in pulmonary vascular resistance did not alter flow significantly.

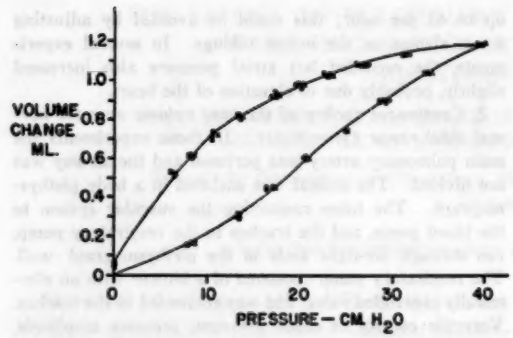

FIG. 2. VOLUME-PRESSURE CURVES OF THE RIGHT LUNG OBTAINED AT VARIOUS BLOOD FLOWS THROUGH THAT LUNG (EXPERIMENT No. 44)

Blood flows through that lung were equivalent to total pulmonary blood flows of 75 (○), 46 (□), 141 (×), and 61 (△) ml. per Kg. body weight per minute. The corresponding pulmonary arterial pressures at the beginning of each run were 27, 22, 32, and 27 cm. H_2O. Lower limb obtained during inflation, upper during deflation. All pressures are final values during the intervals between volume steps.

termined by relating the resistive component of transpulmonary pressure fluctuations to simultaneous rates of air-flow (14).

RESULTS

Measurements during stepwise inflation and deflation

Figure 2 shows inflation and deflation curves of one lung with pulmonary blood flows equivalent to total lung flow rates[6] of 46 to 141 ml. per Kg. body weight per minute. Left atrial pressure was in the normal range. Similar results were obtained in eight dogs on nine occasions. In two of these dogs, the flow through one lung was varied between zero and values equivalent to a total lung flow of 250 and 450 ml. per Kg. body weight per minute, respectively. The pulmonary arterial pressures ranged from 12 to 60 cm. H_2O. The volume-pressure curves did not differ significantly over these ranges of flow.

Contrariwise, when left atrial pressure was elevated to 30 cm. H_2O or more, there was a small but definite change in the volume-pressure loops (Figure 3). At large volumes, both during inflation and deflation, the transpulmonary pressure was slightly higher. In two of these experiments and in a third (not illustrated), there was, however, no change when left atrial pressure was elevated to 15 to 25 cm. H_2O. All changes were immediately reversible unless pulmonary edema developed. In one experiment, in which the left atrial pressure was repeatedly in excess of 40 cm. H_2O for more than 15 seconds, the changes were not reversible; at the termination of the experiment, frothy fluid was found in the lung.

In two experiments the following observation was made: With the lung passively collapsed at the conclusion of a run, a sudden lowering of left atrial pressure was accompanied by further emptying of the lung; *e.g.*, a 225 ml. decrease in volume was recorded when the left atrial pressure was lowered from 55 to 15 cm. H_2O. The change in lung volume was proportional to the change in left atrial pressure. No corresponding increases in lung volume were found when the left atrial pressure was suddenly raised to high levels.

Measurements during continuous volume cycling

In these experiments, the normal tidal range and frequency of breathing were simulated. The end-expiratory airway pressure was maintained at approximately 5 cm. H_2O. The frequency of cycling was 16 per minute. Tidal volumes ranged from 130 to 300 ml.

No change in pulmonary compliance was observed when pulmonary blood flow was varied from 40 to 190 ml. per Kg. body weight per minute. This was studied in four animals. In one experiment, the right lung was excluded by means of a clamp in order to obtain very high flow rates in the left lung; the compliance of the left lung was not significantly changed with blood flow rates equivalent to total lung flow rates of 150 and 370 ml. per Kg. body weight per minute and pulmonary arterial pressures of 20 to 75 cm. H_2O.

The relation between left atrial pressure and pulmonary compliance was studied in three animals, and the results are shown in Figure 4. Pulmonary compliance decreased as left atrial pressure was increased. At left atrial pressures of 50 cm. H_2O the decrease in pulmonary compliance was 20 to 30 per cent. Pulmonary compliance returned to control levels simultaneously with reduction of left atrial pressure.

[6] Assuming a normal right/left flow ratio of 60/40.

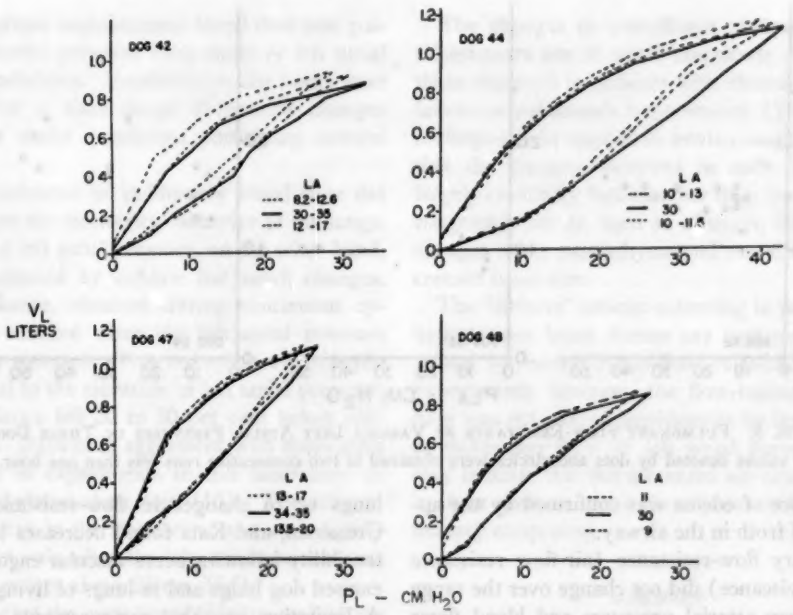

FIG. 3. VOLUME-PRESSURE CURVES OF ONE LUNG OF FOUR ANIMALS AT VARIOUS LEFT ATRIAL PRESSURES

Control curves (interrupted lines) were obtained before and after the curves at high atrial pressure (solid line).

In two experiments, the cycling rate of the respirator was increased from 16 to 120 c.p.m. Pulmonary compliance values were closely similar at the two frequencies (less than 10 per cent difference), both at low and high left atrial pressures.

As has been pointed out, compliance changes occurring with transient elevation of left atrial pressure were immediately reversible. In two experiments, pulmonary edema was intentionally produced by repeated short-term elevations of left atrial pressure. Pulmonary compliance decreased progressively from more than 0.035 to less than 0.005 L. per cm. H_2O and remained low despite reduction of left atrial pressure to control levels.

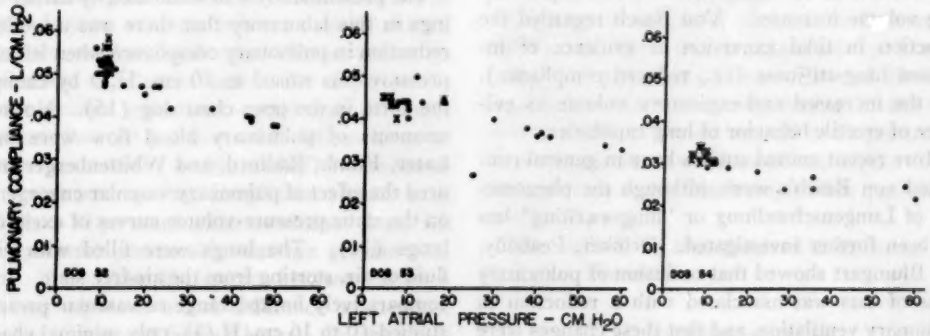

FIG. 4. RELATION BETWEEN LEFT ATRIAL PRESSURE AND LUNG COMPLIANCE IN THREE DOGS

Values denoted by crosses were obtained first; then the left atrial pressures were varied repeatedly over the range of left atrial pressures.

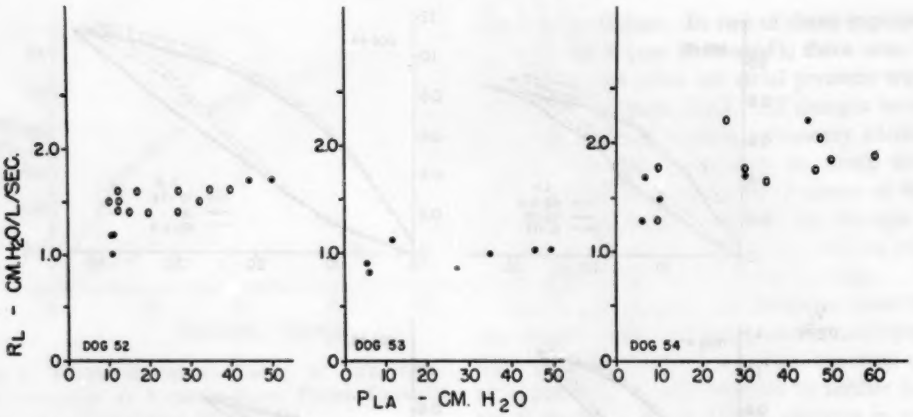

FIG. 5. PULMONARY FLOW-RESISTANCE AT VARIOUS LEFT ATRIAL PRESSURES IN THREE DOGS
The values denoted by dots and circles were obtained in two consecutive runs less than one hour apart.

The presence of edema was confirmed by the appearance of froth in the airway.

Pulmonary flow-resistance (air-flow resistance and tissue viscance) did not change over the range of pulmonary arterial pressures and blood flows studied. With rises in left atrial pressure, pulmonary flow-resistance was unchanged or showed a slight increase (Figure 5).

DISCUSSION

In 1887, von Basch produced acute pulmonary vascular engorgement in living dogs and observed changes in the mechanical behavior of the lungs. In open-chest preparations, ventilation was produced by means of a pump with a fixed pressure amplitude. With congestion the tidal volume decreased, but at the same time the end-expiratory lung volume increased. Von Basch regarded the reduction in tidal excursion as evidence of increased lung stiffness (*i.e.*, reduced compliance), and the increased end-expiratory volume as evidence of erectile behavior of lung capillaries.

More recent animal studies have in general confirmed von Basch's work, although the phenomenon of Lungenschwellung or "lung swelling" has not been further investigated. Drinker, Peabody, and Blumgart showed that occlusion of pulmonary veins of cats was associated with a reduction in pulmonary ventilation, and that these changes were immediately reversible (8). It is not possible, however, to conclude from their data whether these changes related to altered elastic behavior of the lungs or to changes in flow-resistance. Mack, Grossman, and Katz found decreases in lung distensibility following acute vascular engorgement in excised dog lungs and in lungs of living dogs (9). A limitation was that measurements were made over extremely limited ranges of lung volume increase (25 ml. in excised lungs, 60 ml. *in vivo*), and were initiated from the passively collapsed state. Heyer, Holman, and Shires found marked reduction in lung distensibility following rapid infusion of saline solution (10). The lungs of these animals were found post mortem to be hemorrhagic and heavy, and it is not possible to dissociate from their results the possible contribution of pulmonary edema. None of the above studies included measurements of vascular pressures or rates of blood flow through the lungs.

The present study was stimulated by earlier findings in this laboratory that there was a moderate reduction in pulmonary compliance when left atrial pressure was raised to 70 cm. H_2O by clamping the aorta in the open-chest dog (15). No measurements of pulmonary blood flow were made. Later, Frank, Radford, and Whittenberger measured the effect of pulmonary vascular engorgement on the static pressure-volume curves of excised cat lungs (11). The lungs were filled with either fluid or air, starting from the air-free state. In the comparatively limited range of vascular pressures studied (0 to 16 cm. H_2O), only minimal changes in the pressure-volume behavior were found.

In the present study, it was possible to examine separately in the living animal the mechanical ef-

fects of changes in pulmonary blood flow and pulmonary arterial pressure from those of left atrial pressure variations. Furthermore, the lungs were studied over a wide range of volume changes as well as under conditions simulating normal breathing.

Large variations of pulmonary blood flow did not influence the mechanical behavior of the lungs. Elevation of left atrial pressure, on the other hand, was accompanied by definite but small changes. The compliance, obtained during continuous cycling, was reduced when the left atrial pressure was acutely elevated. This reduction was roughly proportional to the elevation of left atrial pressure, and compliance fell 20 to 30 per cent below control values. Results in agreement with these were later found in experiments in this laboratory on anesthetized closed-chest dogs (16). The volume-pressure curve in the static runs was also slightly changed, but only when the left atrial pressure was elevated to values above 25 cm. H_2O.

The fact that the compliance is influenced by the pulmonary venous pressure but not by the pulmonary arterial pressure suggests that the effect is due either to distension of the veins or capillaries or to increased filtration through the capillary walls. If the latter were the case, this would require an extremely rapid fluid exchange, inasmuch as the compliance returned to control level within 10 seconds after the left atrial pressure had been lowered from 50 to 6 cm. H_2O.

The degree of reduction of pulmonary compliance with pulmonary venous engorgement observed is of a considerably smaller magnitude than that reported by Bondurant, Hickam, and Isley in normal human subjects following acute central congestion, produced either by inflation of an anti-gravity suit or submersion in water (7). In the present studies, pulmonary compliance decreased not more than 30 per cent when left atrial pressures were increased to 60 cm. H_2O. Bondurant and associates reported compliance reduction of more than 50 per cent under conditions when left atrial pressure presumably was not greater than 45 cm. H_2O. The discrepancy between these results may represent a true difference between the behavior of dog and human lungs. On the other hand, the reliability of esophageal pressure as a measure of intrapleural pressure in circumstances of central vascular congestion is not known.

The changes in compliance observed in these experiments are of small magnitude compared to those observed in patients with chronic congestive failure or pulmonary hypertension (2–6). If our findings can be applied to human lungs, it is likely that the changes observed in such patients are largely caused by factors other than the pulmonary congestion *per se*, such as effusion, other chronic changes in the parenchyma and vessel walls, or increased heart size.

The "asthma" attacks occurring in patients with hypertensive heart disease are supposedly accompanied by increased air-flow resistance. In our experiments, however, the flow-resistance of the lung was not altered significantly by large changes in pulmonary flow or left atrial pressure. This may indicate that the increased air-flow resistance in these patients is not directly caused by the pulmonary congestion.

SUMMARY

Blood flow to one or both lungs and the left atrial pressure were varied independently. The mechanical properties of the lungs were studied. This was done during stepwise excursions of the lung between passive collapse and a large volume, and during rapid cycling in the normal tidal range of lung volume.

Changes of pulmonary blood flow between 0 and 450 ml. per Kg. body weight per minute, and pulmonary arterial pressures between 12 and 60 cm. H_2O, with the left atrial pressure maintained almost constant, did not significantly alter the mechanical behavior of the lung.

Elevation of left atrial pressure to between 30 and 40 cm. H_2O was accompanied by a small change of the volume-pressure curve of the lung. Compliance, observed during cycling in the tidal volume range, decreased only 20 to 30 per cent at left atrial pressures of 50 to 60 cm. H_2O. These changes were reversible, except when pulmonary edema occurred.

The relation of the observed data to clinical findings is discussed.

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THE BINDING OF CATIONS BY CHONDROITIN SULFATE

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The polyelectrolyte nature of chondroitin sulfate and related polysaccharides suggests they may bind cations. Since chondroitin sulfate is an extracellular component of mammalian connective tissue, the binding properties of this polyelectrolyte for cations may be of significance in the distribution and storage of extracellular cations. In disease states in which sodium is known to play a major role its storage and distribution may be altered as a result of changes in extracellular polysaccharide metabolism. The purpose of the present study is to determine the binding properties of chondroitin sulfate polyanions for the cations sodium, potassium, and calcium. The results indicate that sodium and potassium are bound to chondroitin sulfate to about the same extent, and that binding of calcium is somewhat greater.

Chondroitin sulfate is a salt with a molecular weight of about 20,000 to 30,000 (1), which is often isolated with sodium or potassium as its cation. It is a polymer consisting of about 50 repeating units of the kind represented in Figure 1 (2). If sodium chondroitin sulfate were completely ionized, the resulting polyanion could carry a total negative charge of 100 units per molecule. In the dry neutral salt this charge is balanced by 100 equivalents of cation such as sodium or potassium. Such cations are called counterions. During the past few years, study of several different types of synthetic polyelectrolyte salts has suggested that their ionization in solution is in effect not complete and that part of the total number of counterions may be bound to the polyion (3–9).

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EXPERIMENTAL

Chondroitin sulfate, isolated from beef nasal cartilage, was prepared as the crystalline calcium salt by methods previously described (10). This calcium salt was converted to the sodium or potassium salt by the use of the sodium or potassium form of Dowex-50. Chondromucoprotein as the potassium salt and desulfated chondroitin sulfate as the methyl ester were prepared by methods described elsewhere (11, 12). For convenience these substances will be referred to as Na_2ChS, K_2ChS, or CaChS for the chondroitin sulfate salts, K_2CMP for the chondromucoprotein salt, and MeCh for the methyl ester of desulfated chondroitin sulfate. Polyion concentrations are given as period weights per liter. In each case these weights are the weight of the dry substance that on hydrolysis yields one gram molecular weight of galactosamine. Analytical data are given where the preparations are described (10–12).

Chondroitin sulfate as its sodium, potassium, or calcium salt was dissolved in distilled water to a concentration of 5 mg. per ml. Such solutions in amounts of 5 ml. were pipetted into cellophane bags which had previously been washed in distilled water. A few experiments were set up with the chondroitin sulfate at lower concentrations. Solutions of K_2CMP were transferred by weight rather than by volume because of the high viscosity of the solutions in water in the absence of added salt. The cellophane bags were tightly double-knotted at each end and placed in glass vials. To each vial was added 10 ml. of a solution of the corresponding sodium, potassium, or calcium chloride at a concentration in the range 1 to 140 μEq. per ml. On any given day each concentration of chondroitin sulfate salt with each of the concentrations of its corresponding chloride were run in duplicate. The vials were tightly stoppered with screw caps and placed in a slow rocker or in a revolving wheel for gentle agitation at 4° C. for 24 hours; a few experiments were run for 48 to 96 hours.

The volume of each solution was measured and the solution analyzed for the appropriate cation and chloride. Values of pH were always between six and seven, and no appreciable difference was found between the inside and outside solutions. Sodium and potassium were determined by an internal standard flame photometer. Calcium was determined by titration with ferrous ammonium sulfate after precipitation with oxalate and oxidation by ammonium tetranitratocerate (13). Chloride determinations were carried out by potentiometric titration with silver nitrate (14).

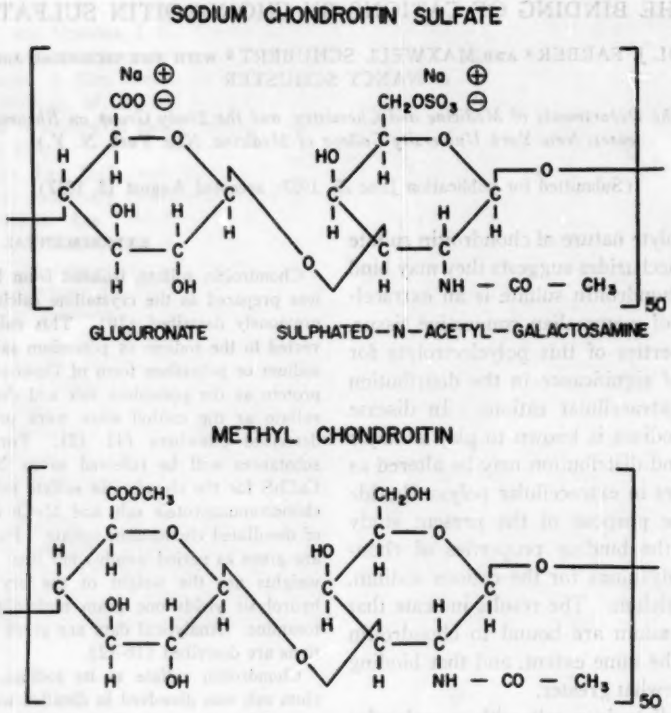

FIG. 1. STRUCTURE OF THE REPEATING UNITS OF CHONDROITIN SULFATE AND METHYL CHONDROITIN

TABLE I

*Values of sodium and chloride ion concentrations inside and outside the bag after equilibration for the indicated hours at 5° C.**

Dialysis time	Na^+ outside	Cl^- outside	Na^+ inside	Cl^- inside	$Na^+_i - Cl^-_i$
hours	*μEq./ml.*	*μEq./ml.*	*μEq./ml.*	*μEq./ml.*	*μEq./ml.*
24	40.0	40.5	56.4	36.9	19.5
	40.1	40.3	56.4	36.9	19.5
	41.8	41.2	54.0	36.3	17.7
	41.3	41.2	53.0	36.6	16.4
	39.7	41.7	54.1	36.8	17.3
	39.7	41.5	53.7	37.1	16.6
	42.2	40.6	53.1	35.6	17.5
	41.5	41.3	54.0	36.9	17.1
	40.2	41.0	53.8	36.6	17.2
48	41.2	41.0	53.9	35.9	18.0
	41.8	41.0	53.5	35.9	17.6
	40.4	41.0	52.8	36.6	16.2
	41.2	41.2	53.2	36.6	16.6
72	40.7	40.6	54.7	36.6	18.1
	41.2	41.6	51.8	37.5	14.3
96	40.6	41.0	52.8	37.2	15.6
	40.3	40.5	53.6	36.5	17.1

* Initial conditions: Inside the bag, 25 mg. Na_2ChS in 5 ml. H_2O (8.7 μperiods per ml.); outside the bag, 10 ml. 0.06 M NaCl.

RESULTS

Table I gives a sample of the experimental results for sodium chondroitin sulfate dialyzed against an initial sodium chloride concentration outside the bag of 0.06 M. The last column, the difference between the sodium and chloride ion concentrations inside the bag at equilibrium, averages 17.2 μEq. per ml. and serves as a check since this should be twice the chondroitin sulfate concentration inside the bag, which is 8.7 μperiods Na_2ChS per ml. Another check is the difference between the values of the sodium and chloride ion concentrations outside the bag, which should be zero. The greatest difference found (Line 5) amounts to 5 per cent of the values measured. Table I also shows that equilibrium is reached in 24 hours and is stable up to 96 hours.

Instead of presenting all of the measurements in detail as in Table I, the data are summarized in Tables II, III, and IV by averaging sets of experiments such as those of Table I. In Table II are the results of experiments with sodium chon-

TABLE II

Mean values for ion concentrations inside and outside the bag at several concentrations of sodium chloride and of sodium chondroitin sulfate (period weight 576)

1	2	3	4	5	6	7	8	9	10	11
			Conc. at equilibrium, *μEq. per ml.*							
Conc. Na_2ChS	Conc. NaCl at start	No. of experiments	Na^+o	Cl^-o	Na^+i	Cl^-i	Free Na^+i	Bound Na^+i	ChS^-	Bound Na^+i
mg./ml.	*μEq./ml.*								*μper./ml.*	*μEq./μper.*
1.0	40	2	28.8	26.8	30.8	26.5	29.1	1.7	2.15	0.79
	60	2	42.0	40.8	43.9	40.4	42.4	1.5	1.75	0.86
	80	2	55.4	53.8	57.6	53.0	56.2	1.4	2.3	0.61
2.5	10	2	7.4	7.3	14.0	5.9	9.1	4.9	4.05	1.21
	20	2	14.2	14.1	20.5	12.3	16.5	4.0	[illegible]	0.98
	30	2	21.0	20.7	26.7	19.1	22.8	3.9	3.8	1.03
	40	2	28.2	27.5	35.1	25.6	30.3	4.8	4.75	1.01
5.0	2.5	4	3.46	2.59	13.4	1.42	6.31	7.1	6.0	1.18
	5.0	5	4.6	4.2	15.7	2.4	8.1	7.6	6.65	1.14
	10	6	8.0	7.8	19.3	5.0	12.4	6.9	7.15	0.97
	20	8	14.8	15.0	26.4	11.4	19.5	6.9	7.5	0.92
	30	4	21.1	21.2	34.2	17.4	25.7	8.5	8.4	1.01
	40	8	27.6	28.0	40.1	24.2	32.0	8.1	7.95	1.02
	60	17	40.8	41.0	53.8	36.6	45.7	8.1	8.6	0.94
	80	16	54.4	54.0	66.4	49.4	59.4	7.0	8.5	0.82
								Weighted average		0.95 (n = 82)

droitin sulfate at the three concentrations given in Column I. In Column 2 are the concentrations of NaCl outside the bag at the start of each experiment. Columns 4, 5, 6, and 7 give the experimentally determined values at equilibrium of the sodium and the chloride outside and inside the bag. In all cases the product of the sodium and chloride concentrations inside the bag is greater than the product of their concentrations outside the bag. Assuming this discrepancy to be due to binding of cation by the chondroitin sulfate anion and that the Donnan equilibrium holds approximately for concentrations instead of ion activities, the data of Columns 4, 5, 6, and 7 can be used to

TABLE III

Mean values for ion concentrations inside and outside the bag at several concentrations of potassium chloride and potassium chondroitin sulfate (period weight 608)

1	2	3	4	5	6	7	8	9	10	11
			Conc. at equilibrium, *μEq. per ml.*							
Conc. K_2ChS	Conc. KCl at start	No. of experiments	K^+o	Cl^-o	K^+i	Cl^-i	Free K^+i	Bound K^+i	ChS^-	Bound K^+i
mg./ml.	*μEq./ml.*								*μper./ml.*	*μEq./μper.*
2.5	20	2	13.9	13.8	20.0	12.6	15.2	4.8	3.7	1.30
	30	2	20.8	20.3	26.6	18.9	22.3	4.3	3.85	1.12
	40	2	27.6	26.9	33.8	24.9	29.8	4.0	4.45	0.90
5.0	1	2	1.52	1.08	10.8	0.38	4.3	6.5	5.21	1.25
	2	2	2.14	1.88	14.2	0.75	5.4	8.8	6.72	1.31
	5	2	4.36	4.17	16.0	2.05	8.9	7.1	6.97	1.02
	10	2	7.32	7.53	18.6	4.77	11.6	7.0	6.91	1.01
	20	8	15.1	14.3	26.1	11.5	18.8	7.3	7.3	1.00
	30	2	21.3	21.0	32.0	18.2	24.6	7.4	6.9	1.07
	40	8	28.2	27.7	39.6	25.0	31.2	8.4	7.3	1.15
	60	4	41.3	40.7	52.5	37.6	44.7	7.8	7.45	1.05
	80	6	55.4	53.9	66.8	51.1	58.5	8.3	7.85	1.06
	140	2	94.7	94.0	104.8	90.4	98.5	6.3	7.2	0.88
								Weighted average		1.08 (n = 44)

TABLE IV

Mean values for ion concentrations inside and outside the bag at several concentrations of calcium chloride and one of calcium chondroitin sulfate (period weight 570)

1	2	3	4	5	6	7	8	9	10	11
			Conc. at equilibrium, μEq. per ml.							
Conc. CaChS	Conc. $CaCl_2$	No. of experiments	$Ca^{++}o$	Cl^-o	$Ca^{++}i$	Cl^-i	Free $Ca^{++}i$	Bound $Ca^{++}i$	ChS^-	Bound $Ca^{++}i$
mg./ml.	μEq./ml.								μper./ml.	μEq./μper.
5.0	20	8	12.5	12.4	25.4	11.8	13.8	11.6	6.8	1.71
	40	7	23.4	23.8	37.0	22.8	25.8	11.5	7.1	1.62
	60	8	35.7	35.1	48.8	33.7	38.8	10.0	7.55	1.32
	80	10	46.2	46.9	62.0	45.7	48.6	13.4	8.15	1.64
								Weighted average		1.58 (n = 33)

calculate the amount of sodium bound. The first assumption can be represented by the equation:

$$Na^+i \text{ free} + ChS^- \rightleftarrows (Na^+i \text{ bound} \cdot ChS^-) \quad (A)$$

The second assumption permits calculation of Na^+i free from the Donnan relation:

$$Na^+i \text{ free} = \frac{Na^+o \times Cl^-o}{Cl^-i} \quad (B)$$

where Na^+i free is the concentration of unbound sodium in the inside solution, and Na^+o, Cl^-o, and Cl^-i are the concentrations of sodium and chloride outside and chloride inside, respectively (Columns 4, 5, and 7). Finally, since the analytically determined values of sodium in Column 6 represent the total sodium (bound plus free) inside the bag, the bound sodium may be calculated from the relation:

$$Na^+i \text{ bound} = Na^+i \text{ total} - Na^+i \text{ free} \quad (C)$$

Column 8 of Table II gives the values of Na^+i free calculated by equation (B), and Column 9 gives values of Na^+i bound calculated by the use of equation (*C*) both in μEq. per ml.

In order to relate experiments done at various concentrations of chondroitin sulfate and at the same time to correct for the small changes in volume inside the bag due to shifts of water, the bound sodium inside the bag has been calculated per period of chondroitin sulfate by dividing the values of Column 9 by the periods per ml. of chondroitin sulfate. This figure is obtained from the relation:

$$ChS = 1/2\ (Na^+i - Cl^-i)$$

Column 10 contains values of the concentration at equilibrium of chondroitin sulfate as μperiods per ml, and Column 11 the values of Na^+i bound as μEq. per μperiod of chondroitin sulfate. At several different concentrations of both chondroitin sulfate and sodium chloride these values appear to be constant and to average 0.95 equivalent of sodium per period of chondroitin sulfate. This amounts to a 47 per cent neutralization of the charge the polyion would have if it were completely ionized.

Similarly, the data of Tables III and IV show that potassium and calcium ions are also bound. The binding of potassium seems to be slightly higher than that of sodium. Calcium binding, however, is higher, 1.6 μEq. per μperiod. This value for calcium represents 80 per cent neutralization of the charge the chondroitin sulfate would have if it were completely ionized.

There is evidence that chondroitin sulfate in cartilage exists as a component of a mucoprotein (11). For studies on the physiology of cartilage and probably of connective tissue, the binding of cations by the mucoprotein would be of more significance than the binding of cations by chondroitin sulfate. In Table V are data obtained by equilibrium dialysis of potassium chloride against the potassium salt of chondromucoprotein. Potassium binding seems to be slightly lower than occurs with chondroitin sulfate.

The analysis of the data presented is based on the assumption that the Donnan expression of the equilibrium is valid for the system studied if concentrations are used in the equation (*B*). The equilibrium is more correctly expressed as a product of activities:

$$a_{Na^+i} = \frac{Na^+o \times Cl^-o \times \gamma o^2}{Cl^-i \times \gamma i} \quad (D)$$

In this equation $^{a}Na^{+}i$ is the activity of sodium in the inside solution, γo is the activity coefficient of either the sodium or the chloride ion outside the bag, which is simply the value in sodium chloride solution (15). The activity coefficient of the chloride ion inside the bag, γi, is assumed to be the same as in sodium chloride solution since there is probably no interaction between the chloride ion and the chondroitin sulfate. Evidence that polyions do not affect the activity coefficients of by-ions (small ions of the same kind of charge as that of the polyion) has been discussed by Kagawa and Katsuura (16). Since the concentrations of Cl^{-} inside and outside the bag are nearly equal, γi and γo will be practically equal and the equation (*D*) simplifies to (*E*):

$$^{a}Na^{+}i = \frac{Na^{+}o \times Cl^{-}o \times \gamma o}{Cl^{-}i} \qquad (E)$$

This gives a simple means to calculate the activity of the sodium ion inside the bag. Assuming the concentrations of sodium ion inside the bag are given by the values in Column 6, Table II, it is now possible to calculate the activity coefficients of the sodium inside the bag, γ $Na^{+}i$, in the presence of chondroitin sulfate from the relation:

$$\gamma Na^{+}i = \frac{^{a}Na^{+}i}{Na^{+}i \text{ total}} \qquad (F)$$

Such calculations have been made for both sodium and potassium ions and the results are plotted as Curve A in Figure 2. This curve has an unusual shape, showing a very sharply decreasing activity coefficient as the concentration of sodium or potassium decreases from 0.03 toward 0.01 *M*. The more familiar simple alkali metal salts over this range show a different curve, with a rising activity coefficient which approaches a value of one as the concentration approaches zero (17). Curve B of Figure 2 is such a curve. It is plotted through the circles which represent the activity coefficients of sodium or potassium ions in solutions of their chlorides at the concentrations represented by the abscissae (15).

It appears that there are two explanations for the results contained in Tables II, III, IV, and V. The first is that the activity coefficient of the cation inside the bag is depressed due to the presence of the polyion and leads to Curve A of Figure 2. The second is that some cations inside the bag are bound to the polyanion and lead to the calculated values of Columns 9 and 11 of Tables II to V. The first explanation is thermodynamically correct but gives no reason why activity coefficients should be progressively more depressed at lower salt concentrations. The second explanation suggests a mechanism for the depression of the activity coefficients. In fact, if the activity coefficients are calculated using the free sodium ion inside the bag (Column 8, Table II) instead of the total sodium ion (Column 6, Table II), then the γ Nai turns out to be close to that in simple NaCl solution as shown by using the following equation:

$$\gamma Na^{+}i = \frac{^{a}Na^{+}i}{Na^{+}i - 7.5} \qquad (G)$$

This simply expresses the proposition that the significant sodium ion concentration inside the bag is not $Na^{+}i$, the value of Column 6, Table II, which

TABLE V

Mean values for ion concentrations inside and outside the bag at several concentrations of potassium chloride and one of the potassium salt of chondromucoprotein (period weight 800)

1	2	3	4	5	6	7	8	9	10	11
			Conc. at equilibrium, $\mu Eq.$ per ml.							
Conc. K_3CMP	Conc. KCl at start	No. of experiments	$K^{+}o$	$Cl^{-}o$	$K^{+}i$	$Cl^{-}i$	Free $K^{+}i$	Bound $K^{+}i$	$CMP^{=}$	Bound $K^{+}i$
mg./ml.	$\mu Eq./ml.$								$\mu per./ml.$	$\mu Eq./\mu per.$
5	10	4	7.84	7.56	15.6	5.82	10.2	5.4	4.89	1.10
	20	5	14.4	14.0	22.9	11.7	17.2	5.7	5.6	1.02
	30	2	21.3	21.1	29.3	18.0	25.0	4.3	5.65	0.76
	40	10	27.7	27.4	36.0	24.4	30.5	5.5	5.8	0.95
	50	2	33.9	33.1	44.1	29.6	37.9	6.2	7.25	0.86
	60	4	40.9	40.2	48.9	37.2	44.2	4.7	5.85	0.80
	80	5	54.8	53.1	62.0	50.2	58.0	4.0	5.9	0.68
								Weighted average		0.90 (n = 32)

TABLE VI

Mean values for ion concentrations inside and outside the bag at several concentrations of potassium chloride and with methyl chondroitin (5 mg. per ml.) inside the bag

Conc. KCl at start	No. of experiments	Conc. at equilibrium, μEq. per ml.				$\frac{K^+_o \times Cl^-_o}{K^+_i \times Cl^-_i}$
		K^+_o	Cl^-_o	K^+_i	Cl^-_i	
μEq./ml.						
20	2	14.6	13.8	14.8	13.0	1.05
40	2	28.0	26.3	28.2	25.9	1.01
60	2	40.5	39.3	42.1	38.7	0.98
80	2	54.1	53.2	54.4	52.1	1.02

is the analyzed total concentration of Na^+ inside the bag, but this amount diminished by 7.5, the average number of μEq. per ml. bound to the chondroitin sulfate (the average of the last eight values of Column 9, Table II). Plotting these values of γNa_i against $(Na^+_i - 7.5)$, as well as the corresponding set from the data of Table III for potassium, gives the upper set of solid points of Figure 2, which rather closely follow Curve B except at the dilute end of the curve where errors are greatest. The closeness with which the upper set of solid points fits Curve B over the whole range of concentration is a justification for the assumption that the amount of sodium or potassium ion bound is practically constant (7.5 μEq. per ml.) over the range of these studies.

As a general check on the method and approach used to study the binding problem, an experiment was conducted with a derivative of chondroitin sulfate containing no ionic groups, the methyl ester of desulfated chondroitin sulfate. In this compound the carboxylate group is methylated and the ester sulfate group is replaced by a hydroxyl group (Figure 1). The results summarized in Table VI show that at equilibrium the potassium ion concentrations inside and outside the bag are equal, and the product of the potassium and chloride ion concentrations inside the bag is equal to the product of their concentrations in the outside solution. Not only is there no binding of cations by the methyl chondroitin but, since methyl chondroitin is a non-diffusible, non-charged particle, there is no Donnan effect either.

DISCUSSION

The data of this study are in general agreement with that of others who used different methods on synthetic polyelectrolytes. Huizenga, Grieger, and Wall (3), using solutions of sodium polyacrylate in transference cells, found that the polyelectrolyte bound sodium ions in amounts up to 60 per cent of the total sodium ions present. Wall and Doremus (4) found the fraction of sodium bound decreased with polymer concentration, that the extent of binding was independent of temperature in the range of 0° to 42° C., and that potassium seemed to be bound to the same extent as sodium. Applying the same method to sodium polyphosphate, they concluded counterions could be bound to the extent of 70 per cent (5). For the divalent cation, Wall and Eitel (6) found binding to partially neutralized polyacrylate occurred to the extent of 70 per cent. Oth and Doty (7), from conductance measurements, estimated the effective charge of the polyion of sodium polymethacrylate solutions to be 60 per cent of the total charge that the polyion would have if ionization were complete. The shapes of conductance-concentration curves for a synthetic polycationic salt led Fuoss (8) to conclude that binding of a considerable fraction of the counterions occurred at higher polyion concentration.

The above studies were not made on systems at equilibrium. Kagawa and Katsuura (9) did use an equilibrium system and measured silver ion activities in cells containing carboxymethylcellulose. They found silver ion activity coefficients "depressed to an extent never experienced in simple electrolyte solutions." At constant carboxymethylcellulose the silver ion activity coefficient decreased with decreasing concentration of silver in a concentration range where the reverse would have been expected. This is similar to the trend of Curve A, Figure 2.

Little can be stated concerning the specific nature of the binding of sodium, potassium, or calcium to chondroitin sulfate except that it renders part of the cations osmotically inactive. Two kinds of binding of counterions by polyelectrolytes have been considered (18, 19). In the first, the polyelectrolyte molecule in solution is thought to exist as a microvolume in which the polyion is coiled. This microvolume has a high charge density distinct from the adjacent solution from which the polyion is absent and it therefore attracts counterions. This microvolume is sometimes considered as a separate phase distinct from the rest

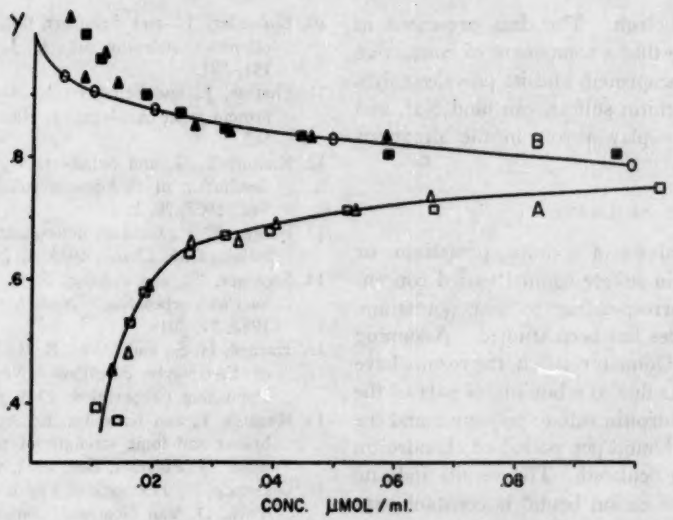

FIG. 2. ACTIVITY COEFFICIENTS OF Na^+ OR K^+ INSIDE THE BAG IN THE PRESENCE OF CHONDROITIN SULFATE

Curve A, values of points calculated from equation (*F*); △ for Na^+; □ for K^+. Curve B, values of points calculated from equation (*G*); ▲ for Na^+; ■ for K^+. The points represented by circles (○) are taken from data for NaCl or KCl given in Harned and Owen (15).

of the solution, and the counterions are not considered to be held in any particular charged sites of the polyion. The second view regards the binding of counterions by polyions to be the result of the formation of specific ion pairs, so a particular counterion is fixed at a particular charged site of the polyion. Either kind of binding would produce an apparent decrease in activity of the counterions.

Ion pairs have been studied for simple ions in media of low dielectric constant such as alcohols (20). Some evidence that ion pair formation can occur between the charged sites of chondroitin sulfate and certain polyvalent complex cobalt cations has been found based on an increase in the ultraviolet absorption of the hexamminecobaltic cation in the presence of chondroitin sulfate (21). In such a system added sodium or potassium chloride can decrease the binding of the complex cobalt cations to the chondroitin sulfate as shown by equilibrium dialysis. If this could be shown to be a competitive effect then it might follow that sodium or potassium ions are also bound to chondroitin sulfate as ion pairs.

The binding of counterions by a polyion in solution is probably different from the ion association that occurs with insoluble ion-exchange resins. An ion-exchange resin is an insoluble polyelectrolyte which must associate an equivalent amount of counterions to maintain electrical neutrality. The state of ionization of the counterions in the insoluble phase of an ion-exchange resin has until recently not been considered (22). The behavior of cations in whole cartilage discussed by Boyd and Neuman (23) is of the ion-exchange type.

The binding of counterions by the anionic polysaccharides of connective tissue probably has physiological consequences. An interesting example is that studied by Joseph, Engel, and Catchpole in their attempt to measure the effect of hormones on the activities of sodium, potassium, and calcium ions in living connective tissues (24).

Increasing evidence has been presented to implicate connective tissue constituents as possibly storing osmotically inactive sodium in certain disease states in man and in experimentally produced conditions in animals (24–29). Tissues chiefly classified as connective tissue in type have been shown to participate in experimental conditions involv-

ing sodium metabolism. The data presented in this study indicate that a component of connective tissue, chondromucoprotein and its polyelectrolyte constituent chondroitin sulfate, can bind Na^+, and this property may play a role in the situations described.

SUMMARY

Equilibrium dialysis of sodium, potassium, or calcium chondroitin sulfate against varied concentrations of the corresponding sodium, potassium, or calcium chlorides has been studied. Assuming the validity of the Donnan relation, the results have been interpreted as due to a binding of part of the cation by the chondroitin sulfate polyanion and the amount of cation bound per period of chondroitin sulfate has been calculated. The results indicate that the amount of cation bound is constant over the concentration range studied, and is approximately 0.9 equivalent per period of chondroitin sulfate for sodium or potassium, and 1.3 equivalents per period for calcium. The mucoprotein of cartilage also bound 0.9 equivalent of potassium per period. Bound cation in the present discussion means osmotically inactive cation.

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SERUM IRON LEVELS IN SIDEROSIS DUE TO HABITUALLY EXCESSIVE IRON INTAKE

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Previous reports from South Africa have drawn attention to the heavy iron intake, and to the high frequency of hemosiderin deposition in the liver and other organs of the indigenous Bantu population (1, 2). In addition, observations in different Bantu population groups have demonstrated elevated levels of serum iron and total iron binding capacity in Bantu adults of both sexes. Saturation is very rarely observed in contrast to findings in idiopathic hemochromatosis (3). In view of these observations it was considered worth while to determine how far serum iron levels reflected the extent of siderosis in a hospitalized population group in this area. Since it is our experience that the severity of hepatic siderosis correlates well with the degree of visceral siderosis elsewhere in the body, the present paper represents our findings in a small series of patients in whom both liver biopsies and serum iron determinations were carried out.

MATERIALS AND METHODS

The liver material for this study came from 26 hospitalized Bantu patients (aged 17 to 66 years) in whom liver biopsies were carried out as necessary adjuncts to diagnosis. Since it was difficult to obtain livers in which siderosis was negligible, one six year old child with minimal siderosis (plus-minus) was added. There were 26 needle biopsies and one surgical biopsy in this series. In Table I, the diseases causing the admission of the patient to hospital are listed. It has been previously shown that there is no association of siderosis to any specific disease (1). The biopsies were examined by routine histopathological methods, but an additional section was stained for hemosiderin by Perl's reaction as described by Lillie (4).

Serum iron levels were determined as previously described (3). Patients in whom a viral hepatitis was suspected clinically were excluded in view of the alterations in serum iron levels known to occur in this condition. In all, a total of 27 subjects (14 males and 13 females) were available for study, but as the data obtained gave significant results it was considered unnecessary to extend the series.

Since these liver biopsies had been obtained for diagnostic purposes it was not possible to determine the degree of siderosis by chemical methods. The degree of hemosiderin deposition was therefore determined subjectively by one observer who was unaware of the serum iron levels. All biopsies were graded when first examined and again at the end of the study after re-staining in order that grading should be as uniform as possible. Siderosis was graded from 0 to 3 plus, according to the degree of hemosiderin deposition in the liver cells (fL) and Kupffer cells (fK), separately, and also according to the total liver deposits (fT). The portal tracts were not considered separately as previous experience indicated that deposits here were variable, being dependent on the degree of portal fibrosis.

RESULTS

The results obtained are summarized in Tables I and II. It will be observed that there is a significant correlation between mean serum iron levels and the presence or absence of siderosis in the liver, especially as determined by the degree of deposition in the Kupffer cells (fK) or in the liver as a whole (fT). The serum iron levels in cases in which the deposits are described as 0 or plus-minus were definitely lower than those in cases with heavily siderotic livers. It was observed, however, that once significant iron deposits were present in the liver no correlation was apparent between the degree of siderosis and mean serum iron levels. For statistical purposes, therefore, the 0 and plus-minus groups were grouped together as were also the 1 plus to 3 plus groups.

In no liver in which hemosiderin was absent (0), of which there were five in the series, did the serum iron levels rise above normal (180 μg. per cent) (Table I). On the other hand, in 3 of the 17 livers in which the iron deposits in the liver were graded 1 plus to 3 plus, the serum iron levels lay below 180 μg. per cent., *i.e.*, Patients 11, 18 and 19 (Table I).

In only one case in the current series was anemia

TABLE I

Serum iron levels, degree of hepatic siderosis, and primary disease causing admission of patient to hospital

Subject	Sex	Age	Disease	Serum iron μg. %	Degree of liver siderosis		
					fL	fK	fT*
1	F	34	Hypochromic anemia	64	0	0	0
2	F	22	Amoebiasis	88	0	0	0
3	M	28	Splenic vein thrombosis	136	0	0	0
4	F	30	Undiagnosed	149	0	0	0
5	M	21	Tuberculosis of spine	176	0	0	0
6	F	6	Malnutrition	60	+	±	±
7	M	36	Hilar lymphadenopathy	125	+	±	±
8	F	23	Chiari's syndrome	167	±	±	±
9	F	22	Pellagra and malnutrition	244	±	0	±
10	F	30	Gonococcal arthritis	264	+	±	±
11	M	47	Hepatic carcinoma	160	+	+	+
12	F	40	Obesity	201	+	+	+
13	F	60	Diabetes mellitus	274	+	++	+
14	F	18	Pellagra and malnutrition	444	+	+	+
15	M	48	Cirrhosis	243	++	++	++
16	F	34	Pellagra and malnutrition	304	+++	+	++
17	M	40	Pellagra and malnutrition	324	+	+++	++
18	M	64	Carcinoma of pancreas	120	++	+++	+++
19	F	43	Pellagra and malnutrition	123	+++	+++	+++
20	M	48	Cirrhosis	201	+++	+++	+++
21	M	31	Tuberculous peritonitis	250	+++	+++	+++
22	M	52	Pellagra and malnutrition	250	++	+++	+++
23	M	42	Diabetes mellitus	250	++	++	+++
24	M	35	Pellagra and cirrhosis	274	+++	+++	+++
25	M	43	Hypertension	385	+++	+++	+++
26	M	44	Pellagra and malnutrition	434	+++	+++	+++
27	F	55	Congestive cardiac failure	490	+++	+++	+++

* The discrepancy which sometimes exists between fT, fL, and fK is due to the fact that fT also takes into consideration the hemosiderin deposits in the portal triads.

TABLE II

Correlation of mean serum iron levels and degree of hepatic siderosis

Degree of siderosis*	Number of subjects	Serum iron, μg./100 ml.			
		Mean	Range	Standard deviation of mean	"t"†
A. In liver cells (fL)					
0 and ±	7	146.3	64–244	22.4	3.29
+ to +++	20	258.8	60–490	25.9	
B. In Kupffer cells (fK)					
0 and ±	10	147.3	60–264	21.8	3.82
+ to +++	17	278.1	123–490	26.4	
C. In total liver (fT)					
0 and ±	10	147.3	60–264	21.8	3.82
+ to +++	17	278.1	123–490	26.4	

* 0 = No hemosiderin deposits present. ± = Minimal hemosiderin deposits. + to +++ = Mild to severe hemosiderin deposits.

† Significance of difference between means calculated according to Hald (6).

of the iron deficiency type present. In this subject the serum iron level was 64 μg. initially at the time of the biopsy and hemosiderin was not demonstrated in the liver. When the degree of siderosis was doubtful (plus-minus), the serum iron levels were variable.

CONCLUSIONS

These findings indicate that high serum iron levels in hospital practice in this region are indicative of increased hemosiderin deposition in the liver of patients who are not suffering from disorders liable to cause elevation of the serum iron levels such as hepatitis, hemolytic anemia, and pernicious anemia in relapse. Occasionally, however, normal serum iron levels may be observed in cases with well marked siderosis. In two of the three siderotic cases with normal serum iron levels in the present series, the patients were seriously ill with advanced neoplastic disease. In view of the findings by Finch and Finch (5) that serum iron levels may fall in patients with hemochromatosis who develop liver carcinoma or severe infection, the probability that the normal serum iron levels in these two patients may be due to concomitant neoplasia must be considered.

The data are insufficient to permit any conclusions regarding the probability of a low serum iron level being found in a siderotic liver.

SUMMARY

Serum iron levels and the severity of siderosis as determined by liver biopsy were correlated in a series of 26 South African Bantu adults and one child derived from a population known to be habituated to an excessively high iron intake. The correlation between mean serum iron levels and the degree of siderosis was highly significant if the patients were divided into groups with and without significant hepatic deposits. There was, however, no correlation between the serum iron levels and the severity of siderosis once significant iron deposition was present in the liver.

ACKNOWLEDGMENTS

We wish to thank Mr. W. Lutz of the University of the Witwatersrand, Johannesburg, for statistical advice, the physicians of the Baragwanath Hospital who permitted us to study their cases, and the Director of the South African Institute for Medical Research for granting us facilities to carry out this research.

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PREGNANE-3α,17α,20α-TRIOL AND PREGNANE-3α,17α,20α-TRIOL-11-ONE EXCRETION BY PATIENTS WITH ADRENOCORTICAL DYSFUNCTION [1]

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The first evidence that adrenocortical hyperplasia involves a fault in steroid metabolism was provided by Butler and Marrian (1–3) with the isolation of large amounts of pregnane-3α,17α,20α-triol from three cases of adrenocortical hyperplasia. This compound was later isolated by Mason and Kepler (4) from three patients with hyperplasia and also from a patient with an adrenocortical tumor, and by Miller and Dorfman (5) from a further case of adrenal hyperplasia. More recently Cox and Marrian (6) isolated it in small quantities from the urine of normal men. The closely related compound pregnane-3α,17α,20α-triol-11-one was isolated from the urine of two patients with adrenocortical hyperplasia by Finkelstein, v. Euw, and Reichstein (7) and more recently by Fukushima, Meyer, Ashworth, and Gallagher (8). The demonstration of the effectiveness of cortisone administration in the relief of symptoms of adrenocortical hyperplasia by Wilkins, Lewis, Klein, and Rosemberg (9) stimulated research into steroid metabolism in this condition.

A method for the quantitative estimation of pregnane-3α,17α,20α-triol and other 17,20-dihydroxy-20 methyl steroids ("acetaldehydogenic steroids") was developed by Cox (10). The acid and enzymic hydrolysis of conjugates of these steroids and their chromatographic separation were investigated by Cox and Marrian (11, 6). Bongiovanni, Clayton, and Eberlein (12, 13), using a reaction based on chromogen production with sulfuric acid, have estimated the excretion of a pregnanetriol-like fraction in adrenocortical hyperplasia, and Zondek and Finkelstein (14, 15) have used fluorometric estimation of a pregnanetriolone-like fraction to distinguish congenital adrenocortical hyperplasia from true hermaphroditism and male pseudo-hermaphroditism. None of these methods is specific for any single compound.

More recently Finkelstein and Goldberg (16, 17) developed an analytical method including paper chromatographic separation for the estimation of pregnane-3α,17α,20α-triol-11-one in human urine. They reported the presence of this compound in the urines of patients with adrenocortical hyperplasia, but not in detectable amounts in the urine of normal people or of patients with adrenal tumors. Because of this finding the latter workers suggested that the presence of pregnanetriolone is characteristic of adrenal hyperplasia and that its detection may be useful in the diagnosis of this condition. By contrast, since pregnanetriol appears to be excreted in greater than normal amounts by patients with tumors of adrenal origin as well as by patients with adrenal hyperplasia, its excretion at abnormally high levels cannot be considered characteristic of adrenal hyperplasia. However, the estimation of pregnanetriolone and pregnanetriol simultaneously is of obvious value in the diagnosis and differentiation of adrenal hyperplasia and adrenal tumors.

A procedure for the simultaneous estimation of pregnanetriol and pregnanetriolone was briefly described by Finkelstein and Cox (18). The present paper describes in detail a method for the identification and estimation of both pregnane-3α,17α,20α-triol and pregnane-3α,17α,20α-triol-11-one suitable for routine clinical use. The results of applying the method to clinical cases are reported and discussed.

METHODS

Reagents. Benzene (thiophene free), chloroform (B.P.), and ethylene dichloride were fractionally distilled before use. Ethanol (absolute) and methanol were refluxed 8 hours with 1 per cent by weight of sodium hy-

[1] This work was supported by a grant and a Senior Research Fellowship (R. I. Cox) from the New South Wales State Cancer Council.

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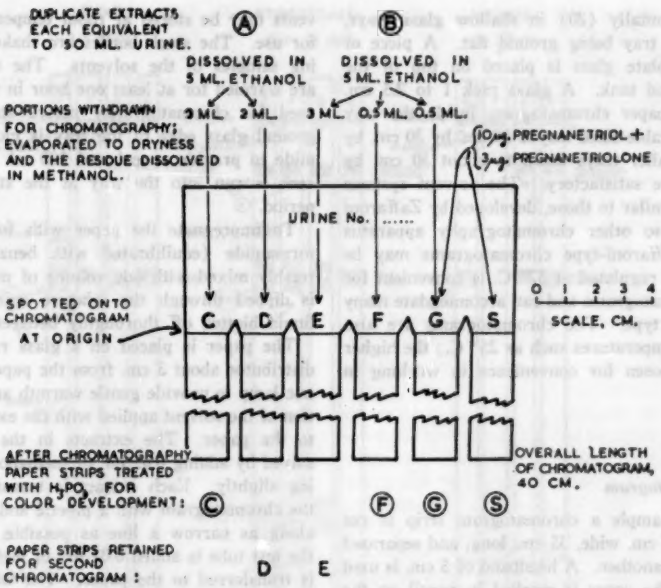
DUPLICATE EXTRACTS EACH EQUIVALENT TO 50 ML. URINE.
A
B
DISSOLVED IN 5 ML. ETHANOL
DISSOLVED IN 5 ML. ETHANOL
PORTIONS WITHDRAWN FOR CHROMATOGRAPHY; EVAPORATED TO DRYNESS AND THE RESIDUE DISSOLVED IN METHANOL.
3 ML. 2 ML. 3 ML. 0.5 ML. 0.5 ML.
10 μg. PREGNANETRIOL +
3 μg. PREGNANETRIOLONE
URINE No. .....
SPOTTED ONTO CHROMATOGRAM AT ORIGIN
C D E F G S
0 1 2 3 4
SCALE, CM.
AFTER CHROMATOGRAPHY PAPER STRIPS TREATED WITH H3PO4 FOR COLOR DEVELOPMENT:
OVERALL LENGTH OF CHROMATOGRAM, 40 CM.
C
F G S
PAPER STRIPS RETAINED FOR SECOND CHROMATOGRAM :
D E

FIG. 1. CHROMATOGRAPHY OF URINE EXTRACTS

droxide, and distilled twice. Formamide, laboratory grade (British Drug Houses Ltd.), was used without purification. Acetic acid, sodium acetate, sodium bicarbonate, sodium hydroxide, anhydrous sodium sulfate, and phosphoric acid were of analytical reagent grade. The β-glucuronidase used was derived from the mollusc *Cellana tramoserica*, a limpet common on sea shores near Sydney (19). Other preparations of β-glucuronidase would be expected to be satisfactory. Pregnane-3α,17α, 20α-triol, m.p. 245 to 247° C.[3] and pregnane-3α,17α,20α-triol-11-one, double m.p. 189° C. and 210° C., were used as reference compounds.

Urine collection. A 24 hour collection of urine over 2 ml. of chloroform is made. The volume is measured.

Hydrolysis procedure. A 100 ml. portion of urine is withdrawn and the pH adjusted to 4.6 with glacial acetic acid. Two ml. of 2M acetate buffer of pH 4.6 are added and, after mixing, the sample is divided into two equal portions (A and B). These samples are placed in a 37° C. water bath and allowed to warm for half an hour. To each is added 5 ml. of chloroform and 1 ml. of the β-glucuronidase preparation containing 2,500 units of enzyme. The samples are allowed to stand at 37° C. for 24 hours. These conditions do not necessarily achieve maximum hydrolysis of the steroid conjugates, but have been found satisfactory for clinical use with this method. Other enzyme preparations may require use of different conditions for hydrolysis.

Extraction procedure. Each of the duplicate samples is extracted twice with 100 ml. of chloroform. The chloroform extracts are washed with 20 ml. N sodium hydroxide and the alkali is drained off as thoroughly as possible. The emulsion at the interface is discarded. The chloroform is washed with 10 ml. of 8 per cent sodium bicarbonate solution, draining off the alkali thoroughly. The chloroform extract is run into a clean funnel and washed with 10 ml. of water twice. The washed chloroform is run into a dry flask containing 2 Gm. anhydrous sodium sulfate and the flask is shaken and allowed to stand for ten minutes. The chloroform is filtered through filter-paper into a 1-liter round bottom flask for evaporation. The evaporation is carried out under reduced pressure on a water bath, making sure that the flask is removed from the bath as soon as the last drop of chloroform has evaporated. The residue is dissolved with slight warming in 3 ml. of ethanol and the ethanol extract transferred with a pipette to a graduated centrifuge tube. The flask is rinsed again twice with 1 ml. portions of ethanol. The volume is made up to 5 ml. and stirred to mix the solutions. The portions shown in Figure 1 are withdrawn from the ethanol extract and evaporated to dryness in test tubes (about 1 cm. in diameter) under a gentle stream of nitrogen while heating on a water bath at about 80° C. The test tubes are withdrawn from the water bath as soon as the extracts are dry. These samples are now ready for chromatography.

Paper Chromatography

Apparatus

Whatman No. 1 paper "for chromatography" is used without any purification. Chromatograms are con-

[3] We are indebted to Professor G. F. Marrian for the gift of a sample of this steroid.

veniently run horizontally (20) in shallow glass trays, the top edge of the tray being ground flat. A piece of three-eighths inch plate glass is placed on top of the tray to form a closed tank. A glass rack 1 to 2.5 cm. high supports the paper chromatogram inside the tray (Figure 2).[4] A suitable sized tray is 50 cm. by 30 cm. by 5 cm., although smaller trays down to about 30 cm. by 20 cm. by 3 cm. are satisfactory. The solvent systems used are those, or similar to those, developed by Zaffaroni and Burton (21), so other chromatography apparatus suitable for the Zaffaroni-type chromatograms may be used. An incubator regulated at 37° C. is convenient for the horizontal chromatograms and can accommodate many trays of the above type. The chromatograms are also effective at lower temperatures such as 25° C.; the higher temperature was chosen for convenience in working in Sydney.

Procedure

(a) *First chromatogram*

For each urine sample a chromatogram strip is cut with six tongues 1.5 cm. wide, 35 cm. long, and separated by 0.5 cm. from one another. A headband of 5 cm. is used (see Figure 1). The paper is marked in pencil on the headband with the reference number and letter as shown in Figure 1.

For the first chromatogram the benzene-formamide system is used. Equal volumes (500 ml.) of benzene and formamide are equilibrated at 37° C., and the layers are separated in a funnel at 37° C.; these equilibrated solvents may be stored at room temperature until required for use. The stock bottles are shaken before withdrawing samples of the solvents. The chromatogram trays are warmed for at least one hour in the 37° C. incubator used for chromatography before starting a run. The ground glass edge of the tray is moistened with formamide to provide a seal and 100 ml. of equilibrated benzene is run into the tray at the start of the warming period.

To impregnate the paper with formamide, 10 ml. of formamide (equilibrated with benzene at 37° C.) is freshly mixed with one volume of methanol. The paper is dipped through the mixture quickly and the excess liquid blotted off thoroughly between filter paper.

The paper is placed on a glass rack with a nitrogen distributor about 3 cm. from the paper surface. An electric lamp to provide gentle warmth and speed up evaporation of the solvent applied with the extracts is placed close to the paper. The extracts in the test tubes are dissolved by adding 0.05 ml. of methanol to each and warming slightly. Each extract is transferred to a strip on the chromatogram with a pipette and the paper is spotted along as narrow a line as possible. To the residue in the test tube is added 0.01 ml. of methanol and this wash is transferred to the paper. The wash is repeated with 0.01 ml. of methanol. To the strip with extract G is added 10 μg. of triol and 3 μg .of triolone in methanol, and to the sixth strip S is added these amounts of pure substances alone. When the methanol has dried off, the chromatogram is placed on the glass rack in the prewarmed tank at 37° C. and the run is started by dipping the end of the paper in the solvent. The chromatogram is allowed to run for 2 to 3 hours when the solvent front should have travelled about 30 cm.

At the end of the run the chromatogram is removed

[4] We are indebted to Dr. S. E. Wright for suggesting the use of this modification of the horizontal chromatographic tank.

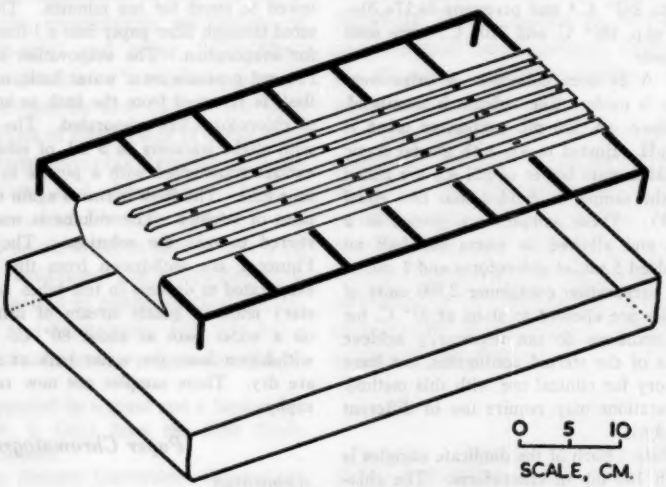

FIG. 2. APPARATUS FOR HORIZONTAL CHROMATOGRAPHY
Glass tray and rack; paper chromatogram in position on rack.

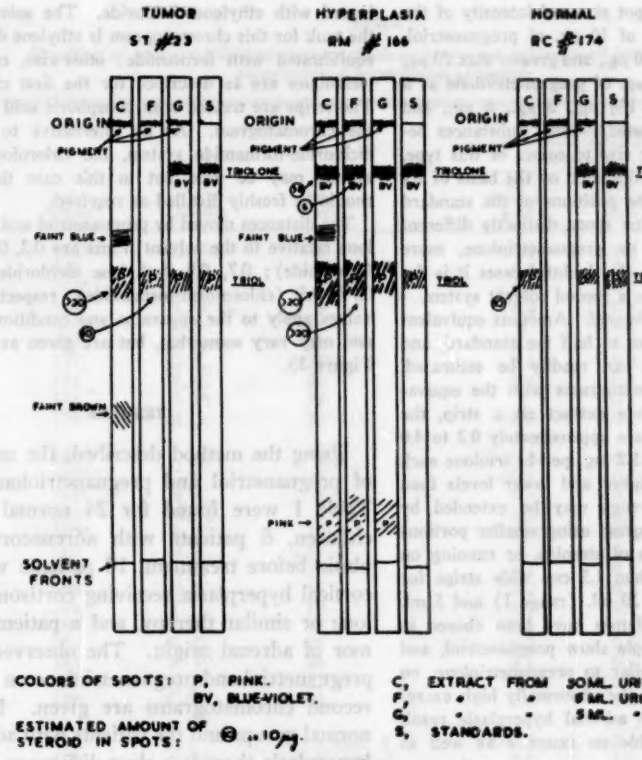

FIG. 3. APPEARANCE OF PAPER CHROMATOGRAMS IN ULTRA VIOLET LIGHT AFTER H_3PO_4 COLOR REACTION

from the tank and suspended from stainless steel clips in an air oven at 90° C. for half an hour, or until dry. An air draft should be provided to remove the outlet gases from the laboratory atmosphere.

Detection of pregnanetriol and pregnanetriolone. The position of the solvent fronts is readily seen from a bluish-white band under the ultra-violet lamp (see below), and this position is marked in pencil on the strips before color development. Strips C, F, G, and S are cut out and dipped through a tray of 70 per cent phosphoric acid (70 ml. phosphoric acid mixed with 30 ml. water). The excess phosphoric acid is blotted off thoroughly twice between filter paper. It is of advantage to use a photographic type squeegee to ensure thorough blotting. The strips are placed immediately on a pre-heated enamel tray in an air oven initially at 85 to 87° C. for 10 plus or minus 1 minute. The paper should show no charring after the heating. The tray is taken out and cooled quickly by playing a jet of tap water against the back of the tray for about half a minute, followed by cooling over another shallow tray containing ice-cold water for a further half minute. Without delay the paper is examined on the tray under an ultra-violet lamp rich in light of 365 to 366 mμ, such as the Research Equipment Corporation RX-15 or Phillips TYP57202F/70.

The position of pregnanetriol is shown by a pink spot and pregnanetriolone by a blue-violet spot. In daylight the position of pregnanetriol is seen as a violet spot, whereas the spot corresponding to pregnanetriolone is not visible. At levels of about 5 μg. and 2 μg. per sq. cm. pregnanetriol and pregnanetriolone are readily seen; as pure compounds they should be detectable down to 1.5 μg. and 0.5 μg. per sq. cm., respectively. The position of the triol and triolone are noted on the strips and corresponding areas on the urine extract strips are examined. If spots corresponding to the steroids are visible in the extract strips, the positions are noted. The positions of added triol and triolone on strip G check whether there is any interference of the urine extract with the chromatography or color development of the two steroids. Colors have been affected when the steroid levels are low and in the normal range. Such interference, if it occurs, is eliminated on chromatography in a second solvent system. The positions of spots are recorded as the limits of the leading and trailing edges (see Figure 3). The amounts of triol and triolone in the extract strips may be

estimated by comparison of spot size and intensity of the standards. With a standard of 10 μg. of pregnanetriol, less than 5 μg., 5 μg., 10 μg., 20 μg., and greater than 20 μg. are estimated levels; with 3 μg. of pregnanetriolone as a standard, less than 1½ μg., 1½ μg., 3 μg., 6 μg., and greater than 6 μg. are estimated. Other substances besides these steroids may give rise to colors of this type, but normally are readily differentiated on the basis of R_F values by comparison with the positions of the standard substances. In some cases blue spots, distinctly different from the blue-violet given by pregnanetriolone, move close to the latter compound. In doubtful cases it is desirable to rechromatograph on a second solvent system.

Amounts of compounds estimated. Amounts equivalent to the standard, to double and to half the standard, and amounts outside this range can readily be estimated. Thus, using the above chromatograms with the equivalent of 5 and 30 ml. of urine extract on a strip, the ranges of steroids estimated are approximately 0.2 to 4.0 mg. per L. triol, and 0.05 to 1.2 mg. per L. triolone each in six steps. In addition, higher and lower levels than these are indicated. The range may be extended by running the second chromatogram using smaller portions of extracts for higher values of steroids, or running on 0.5 cm. wide strips rather than 1.5 cm. wide strips for low values of steroids. The 30 ml. (range 1) and 5 ml. (range 2) urine extract volumes have been chosen in such a way that normal people show pregnanetriol, and very occasionally a spot similar to pregnanetriolone, on range 1, but not on range 2; the abnormally high excretion shown by patients with adrenal hyperplasia result in these steroids being visible on range 2 as well as range 1.

(b) *Second chromatogram*

The strips D and E which have not been dipped in phosphoric acid are cut according to the positions of the steroids found in the strips C, F, G, and S. Normally the solvent fronts should have moved almost equal distances on each strip and a direct comparison of D and E with C, F, G, and S may be made. If the solvent fronts differ from one strip to another, the expected positions of the spots are calculated relative to the movement of the solvent front. An extra margin of 0.5 cm. is allowed at each edge of the spot. Each piece of paper is cut into small pieces about 1 to 2 mm. by 3 to 5 mm. The triol and triolone spots from any one strip are combined. The small pieces of paper are then extracted by soaking and warming in 2 ml. of methanol for a few minutes, decanting off the methanol into a graduated centrifuge tube, and repeating the extraction with 2 ml. of warm methanol twice more. The volume of the extracts is made up to 6 ml. with methanol, and the solutions mixed with a stirring rod. Suitable portions of these extracts, as indicated from the results of the first chromatogram, are evaporated to dryness. The portions are chromatographed on a four-strip paper, the solvent system ethylene dichloride-formamide is used. The paper is impregnated with formamide-methanol 1:1 using formamide equilibrated with ethylene dichloride. The solvent placed in the tank for this chromatogram is ethylene dichloride preequilibrated with formamide; otherwise, conditions and techniques are as described for the first chromatogram. The strips are treated with phosphoric acid after running the chromatogram. As an alternative to the ethylene dichloride-formamide system, the chloroform-formamide system may be used but in this case the chloroform should be freshly distilled as required.

The distances moved by pregnanetriol and pregnanetriolone relative to the solvent fronts are 0.3, 0.08 (benzene-formamide); 0.7, 0.4 (ethylene dichloride-formamide); 0.75, 0.5 (chloroform-formamide), respectively. These values apply to the apparatus and conditions used above and may vary somewhat, but are given as a guide (see Figure 3).

RESULTS

Using the method described, the urinary levels of pregnanetriol and pregnanetriolone shown in Table 1 were found for 24 normal adults and children, 6 patients with adrenocortical hyperplasia before treatment, 10 patients with adrenocortical hyperplasia receiving cortisone, prednisolone or similar therapy, and a patient with a tumor of adrenal origin. The observed values for pregnanetriol and pregnanetriolone on the first and second chromatograms are given. Between the normal groups and the patients with adrenocortical hyperplasia there is a clear difference. In the latter, pregnanetriolone is excreted in relatively large amounts and there is an associated increase in the pregnanetriol levels. The absence of pregnanetriolone with tumors of adrenal origin is suggested by the case reported here and by the two cases of women, one with a virilizing adrenal tumor and the other with an adrenal adenoma, noted by Finkelstein and Goldberg (17). Differentiation of patients with tumors of adrenal origin from adrenocortical hyperplasia on the basis of the pregnanetriolone excretion seems possible. Confirmation of this supposition awaits study of further cases, but it does not appear unreasonable in view of the very definite excretion of pregnanetriolone by every one of twelve patients with adrenocortical hyperplasia (present paper, six cases), its excretion by six hyperplasia patients maintained on cortisone or similar therapy, and its absence in the three tumor cases.

Cortisone treatment is known to decrease the pregnanetriol values (22) and pregnanetriolone values (15). In the above series of results such

TABLE I

Excretion of pregnanetriol and pregnanetriolone by normal subjects and by patients with adrenocortical hyperplasia or tumors

	Urine sample	Subject	Age (yrs.)		Amount of steroid found (mg./24 hrs.) Pregnanetriol 1st chromatogram	Pregnanetriol 2nd chromatogram	Pregnanetriolone 1st chromatogram	Pregnanetriolone 2nd chromatogram
Normal males	162*	K. W.	5		<0.1	<0.1	†	‡
	181	L. E.	5		0.2	0.1	§	§
	163*	R. G.	6		<0.1	<0.1	0.07	‡
	164*	R. O. G.	6		0.2	<0.1	§	§
	160*	J. D.	11		‖	<0.1	§	§
	158	J. T.	23		0.2	0.3	§	§
	157	D. L.	28		0.6	0.8	§	§
	174	R. C.	28		0.3	0.1	§	§
	175	R. P.	28		‖	0.3	§	§
	178	H. S.	36		0.3	<0.2	†	§
Normal females	188	J. M.	2.3		<0.04	<0.04	§	§
	165*	C. S.	5		<0.1	<0.1	§	§
	167*	N. C.	7		0.3	<0.1	0.08	‡
	169*	M. H.	8		0.2	0.1	†	§
	161*	R. K.	13		0.1	<0.1	§	§
	182	H. E.	13		‖	<0.1	§	§
	180	J. E.	15		0.2	0.2	§	§
	186	D. G.	18		<0.2	<0.2	§	§
	184	T.	19		0.6	0.3	§	§
	183	F. B.	27		0.5	0.3	§	§
	176	E. C.	27		0.5	0.2	§	§
	185	R. P.	30		0.1	0.1	§	§
	177	H. S.	32		‖	<0.2	§	§
	179	C. E.	44		0.4	0.2	†	§
Adrenal hyperplasia	190	A.	2 wks.	♀	>0.3	0.4	>0.7	0.9
	194	R. P.	7 wks.	♂	0.6	1.0	0.6	0.6
	149	S. T.	8 wks.	♀	0.2	0.3	0.3	0.5
	101	J. T.	6	♀	3	4	0.2	0.4
	173	C. J.	13	♀	3	3	0.5	0.4
	166	R. M.	16.5	♂	>4	7	1.2	1.0
Adrenal hyperplasia on steroid therapy††	200¶	R. P.	10 wks.	♂	0.05		0.05	
	152	G. S.	1.5	♀	0.3	0.4	0.2	0.2
	156	L. R.	3	♀	0.3	0.3	0.1	0.2
	153	S. S.	4	♀	0.8	0.8	0.4	0.5
	148	B. H.	4	♂	0.4	0.1	§	§
	171**	J. T.	6	♀	0.3	0.3	§	§
	172	M. T.	8	♂	0.1	0.4	§	§
	154	R. Mi.	11	♀	‖	0.3	§	0.07
	197	H. P.	12	♀	1.0	1.3	0.3	0.2
	145††	K. F.	14	♀	>3	4	2.5	1.4
Tumor	23‡‡	S. Ta.	9	♀	3	3	§	§
	189‡‡ (6 mos. post-operative)	S. Ta.			‖	<0.1	§	§

* Hospitalized patients towards end of convalescence.

† Doubtful spot.

‡ Faint spot, not pregnanetriolone (see text).

§ Levels below 50 μg. per 24 hours.

‖ Spot partly obscured by other material or atypical color.

¶ Compare with 194 above, same subject.

** Compare with 101 above, same subject.

†† 17-ketosteroid levels estimated by Institute of Child Health, Sydney, were within normal range except for sample 145; the latter patient showed very variable excretion which here was high at 29 mg. per 24 hours.

‡‡ "Adrenal adenoma" of ovary.

a decrease in indicated in samples 101, 171, 194, and 200, being samples of urine from the same patients before and after cortisone treatment, respectively. It is noteworthy that in samples 152, 156, and 153 from patients with adrenal hyperplasia but on steroid therapy, the 17-ketosteroid excretion was within the normal range, whereas marked amounts of pregnanetriolone were still being excreted.

Presence of other compounds besides pregnanetriol and pregnanetriolone

In some of the chromatograms of the 41 urine extracts reported in this paper, there appeared a number of other spots of a variety of colors apart from the spots of pregnanetriol and pregnanetriolone. Most of the blue colors which have been observed were notably different from the blue-violet color given by pregnanetriolone in the color test. With high levels of pregnanetriol and pregnanetriolone, the presence of other spots did not interfere with the detection and estimation of these steroids on the first chromatogram. However, in extracts from some of the normal subjects, interference was found in the first chromatogram because of the relatively low levels or absence of the steroids. No interference was noted in the second chromatogram even if it occurred on the first.

It should be noted that the spots which are estimated as pregnanetriol and pregnanetriolone are assumed to be these compounds from the colors developed in the phosphoric acid reaction and from their R_P values. However, it is possible that closely related compounds may not be distinguished, especially at low levels of pregnanetriol and pregnanetriolone. Thus, samples 162, 163, and 167 in the normal group showed faint spots corresponding to pregnanetriolone; however, the higher resolution of a 27 hour descending chromatogram showed that these trace substances were not pregnanetriolone. With all the adrenocortical hyperplasia patients, pregnanetriolone showed up very clearly; usually the spot was so intense as to require dilution of fractions for the second chromatogram. Two liters of urine from an adrenocortical hyperplasia patient, R. M., sample 166, were processed by this method except for the use of a sheet of Whatman No. 3 MM paper for chromatography. From the area on the paper corresponding to pregnanetriolone a substance was isolated directly after the second chromatogram. This substance was identified as pregnanetriolone from its infra-red absorption spectrum. The compounds recently isolated by Fukushima, Meyer, Ashworth, and Gallagher (8), allopregnane-3α, 17α,20α-triol and pregnane-3α,11β,17α,20α-tetrol,[5] are completely resolved from pregnanetriol and pregnanetriolone in the benzene-formamide system as used in the method described in this paper (23).

DISCUSSION

The method presented is simple and effective for the detection of pregnane-3α,17α,20-triol and pregnane-3α,17α,20α-triol-11-one in urine, and can be carried out routinely by a technician. No elaborate precautions are required and, in particular, purification of filter paper is not necessary. In tests of the urine of patients with adrenal hyperplasia or adrenal tumors, a single chromatogram is usually sufficient to show the presence and indicate the levels of these steroids. In some cases where there is uncertainty, a second chromatographic separation is required. The minimum amounts detectable are about 100 μg. per 24 hours for pregnanetriol and 50 μg. per 24 hours for pregnanetriolone. The lower levels of pregnanetriol associated with normal adults and especially with normal children sometimes may not be detectable. The tests may be made more sensitive, or larger amounts of urine may be extracted; however, the method as presented is designed primarily to detect abnormally high levels.

The methods which have been in use for estimation of pregnanetriol (10, 12) and pregnanetriolone (15) are not specific for these steroids and demand their prior separation from related or interfering substances. Thus, estimation by means of acetaldehyde produced on periodate oxidation (10) is a general reaction for C21 steroids of the 17,20-dihydroxy-20-methyl series. Substances, in addition to pregnanetriol or pregnanetriolone, giving this reaction have been extracted from urine (6, 8). The method for pregnanetriol based on chromogens produced on reaction with concentrated sulfuric acid (12) requires adequate purification of extracts due to the poor specificity of

[5] Samples of these steroids were generously donated by Dr. T. F. Gallagher.

the reagent. Pregnanetriolone produces an intensely fluorescent compound on heating with concentrated phosphoric acid, but fluorogenic substances other than this compound in neutral urine extracts are excreted by normal people and in increased amounts by patients with adrenal tumors and in late pregnancy (14, 17).

Recently, De Courcy (24) elaborated a method for the estimation of pregnane-3α,17α,20-triols, and Finkelstein and Goldberg (16, 17) for pregnane-3α,17α,20α-triol-11-one. Both methods utilize paper chromatography for the separation of the respective compounds, which are detected by means of fluorescence. It is worth mentioning, however, that in the method of De Courcy only pregnanetriol, but not pregnanetriolone, is visualized by the reagent employed. On the other hand, in the method of Finkelstein and Goldberg, pregnanetriol is destroyed by the hot acid hydrolysis as used for the liberation of pregnanetriolone from its conjugate (25).

Since pregnanetriol produces fluorescence and can be easily detected on paper with the 70 per cent phosphoric acid reagent used for estimation of pregnanetriolone, substitution of the hot acid treatment by enzymic hydrolysis, which liberates both compounds without destroying them, allows for their simultaneous estimation. Moreover, in enzymic hydrolysis, less chloroform extractable impurities are formed than in hot acid hydrolysis, thus making unnecessary the rigorous purification of the extracts described previously for the detection of pregnanetriolone (17). The present method described primarily for clinical use, and obviating the use of precise optical apparatus such as spectrophotometer or fluorimeter, is semiquantitative. Used in conjunction with estimation of acetaldehydogenic (10) or fluorogenic substances (14), it can give precise quantitative measurements of individual compounds such as pregnanetriol or pregnanetriolone and may be used to detect unknown steroids on the chromatograms. Results of such studies will be published elsewhere.

The differentiation between adrenal hyperplasia and adrenal tumor is a problem of clinical importance. Jailer, Gold, and Wallace (26) suggested that the decrease in excretion of 17-ketosteroids on cortisone administration would indicate adrenal hyperplasia. However, in some cases of adrenal hyperplasia of the adreno-genital type the 17-ketosteroids do not readily respond to cortisone therapy (27) (case No. 145, this paper). Moreover, in Cushing's syndrome the differentiation between adrenal hyperplasia and adrenal tumor is hardly possible on the basis of 17-ketosteroid suppression on cortisone administration [*cf.*, Wilkins (28) and Thorn (29)]. Bongiovanni (22) associated high excretion of the pregnanetriol-like fraction with adrenal hyperplasia and observed that on cortisone administration this fraction decreased. In addition to the original isolation of pregnanetriol from the urine of patients with the adreno-genital syndrome by Butler and Marrian (1–3), it has also been isolated from the urine of patients with adrenal tumors (4), and from the urine of normal men (6). High levels of pregnanetriol appear to be associated with adrenal tumors as well as with adrenal hyperplasia, and thus do not allow for differentiation between these two conditions. As to the decrease of pregnanetriol on cortisone treatment, it remains to be proved that such a decrease is more consistent than the corresponding decrease of the 17-ketosteroids. On the other hand, the consistent presence of pregnanetriolone in the urine of patients with adrenal hyperplasia and its apparent absence in detectable amounts from the urine of three patients with adrenal tumor can make it a valuable differential diagnostic aid between these conditions. It has been reported that one patient with the hypertensive form of adrenal hyperplasia (13, 30) excreted no detectable amounts of expected 11-oxy steroids in the urine; although pregnanetriolone was not specifically searched for in this work, it is desirable that data be obtained on the excretion of this steroid by such patients. It is fully realized that a general conclusion on the absence of pregnanetriolone from urine of patients with adrenal tumors is not permissible on the basis of the results obtained on only three cases of tumors examined so far. However, in view of the rarity of this disease and thus of a small chance that our laboratories (located in countries with relatively small populations) will be able to compile a large series of adrenal tumors within a reasonable time, it seems to us that by reporting on the difference in steroid excretion by patients with adrenal hyperplasia and adrenal tumors, research on larger series may be stimulated.

It was suggested in a previous paper (17) that pregnanetriolone is characteristic of adrenal hyper-

plasia. Only six cases of adrenal hyperplasia were investigated. The presence of pregnanetriolone in an additional six patients with adrenal hyperplasia not receiving cortisone therapy and in six patients maintained on cortisone, reported in the present paper, greatly substantiates this supposition. On the other hand, analysis of urine of 24 "normal" people described here, of 37 reported previously (17), and of an additional 25 "normal" persons (31) shows that pregnanetriolone is not detectable in "normal" persons' urine, and if present, it must be in relatively small quantities (less than 50 μg. per 24 hours). High levels are associated with adrenal hyperplasia (see Table I). Children with adrenal hyperplasia of the virilizing type show definite presence of pregnanetriolone, and milligram quantities are excreted by adults with the adreno-genital syndrome due to adrenal hyperplasia.

SUMMARY

A method for the detection and estimation of urinary pregnane-3α,17α,20α-triol and pregnane-3α,17α,20α-triol-11-one suitable for routine clinical use is described.

The results of estimation of the excretion of these steroids by 41 people, including "normal" persons and patients with adrenocortical hyperplasia or tumors of adrenal origin, are presented. Normally, pregnanetriolone is not excreted in detectable amounts but this steroid appears in relatively large amounts in the urine of patients with adrenocortical hyperplasia; pregnanetriolone has not been found in the urine of three patients with tumors of adrenal origin. On the other hand, high pregnanetriol levels appear to be associated with both adrenocortical hyperplasia and with tumors of adrenal origin.

The characteristic finding of pregnanetriolone in cases of adrenocortical hyperplasia should be a useful diagnostic aid.

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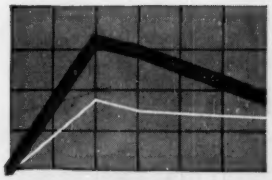

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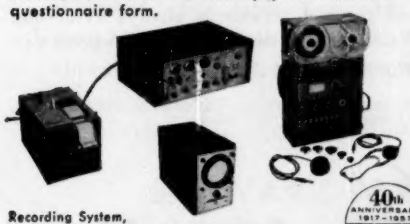

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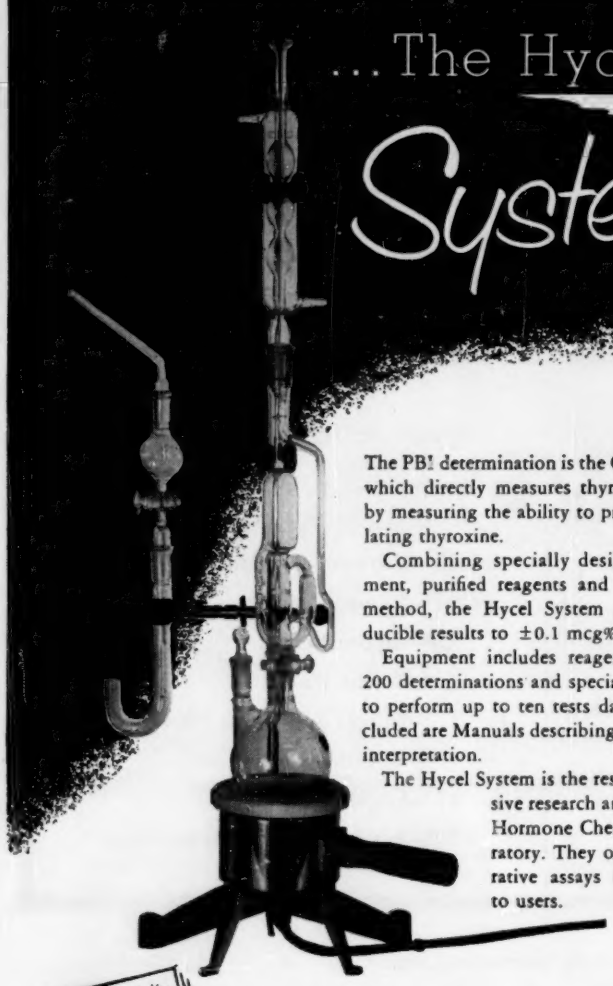

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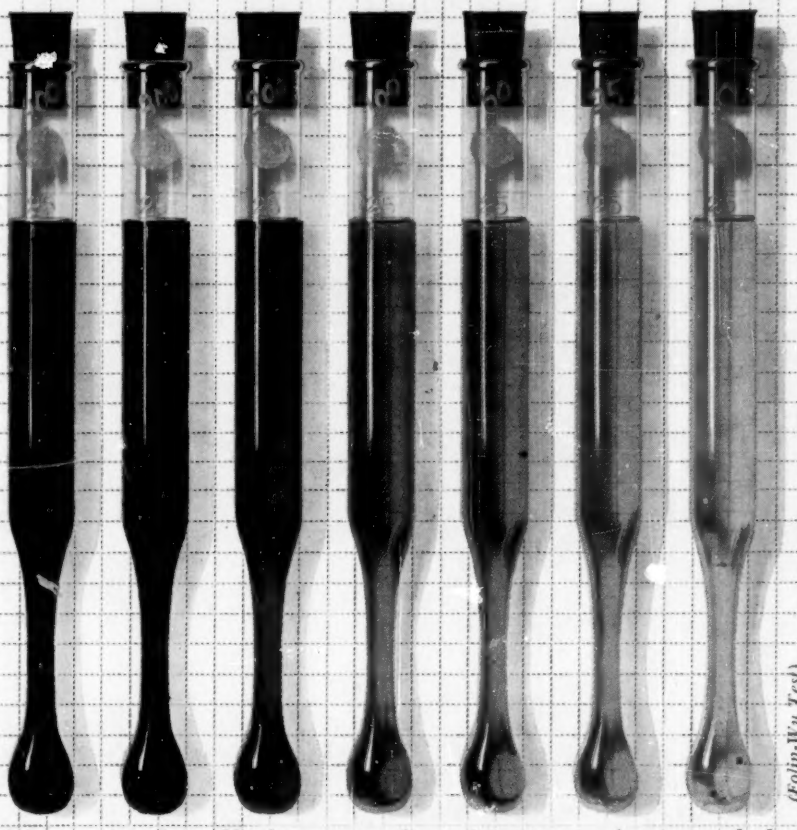

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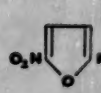

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